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Award Number: DAMD17-02-1-0586

TITLE: Tumor Secreted Autocrine Motility Factor (AMF): Causal Role in an Animal Model of Cachexia

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14. ABSTRACT

Cancer cachexia has three clinical features: 1) loss of appetite (anorexia), 2) nutritional mal-absorption, and 3) muscle and fat wasting caused by tumor-stimulated factors. This project focused on muscle wasting. A number of factors have been proposed to cause cancer cachexia. Lack of progress in the area is unfortunate, given the tremendous benefit patients with advanced cancer would receive from effective treatment of cachexia to improve quality of life and postpone mortality. We proposed that autocrine motility factor (AMF) is released into the bloodstream from cancer sites and stimulates muscle wasting. During the grant period we: 1) Established an animal model in which CHO/AMF tumors caused cachexia in mice; 2) Showed that injection of recombinant AMF protein caused significant weight loss in mice in 24 hours; 3) Solved the 3-D structures of mouse and human proteins; 4) Initiated characterization of the genes involved in muscle protein degradation in response to cachexia in the mouse model with CHO/AMF tumors.

15. SUBJECT TERMS
Cancer cachexia, proteasome, muscle wasting, phosphoglucose isomerase glucose 6-phosphate isomerase, autocrine motility factor

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Tumor Secreted AMF: Causal Role in an Animal Model of Cachexia

John M. Chirgwin Ph.D.   Award DAMD17-02-1-0586

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Pdf of Titus B, Frierson HF Jr, Conaway M, Ching K, Guise T, Chirgwin J, Hampton G,
Theodorescu D. Endothelin axis is a target of the lung metastasis suppressor gene
RhoGDI2. Cancer Res 65:7320-7327, 2005
        Labeled <Titus Can Res 05>

Final Report   John M. Chirgwin Ph.D.

Award DAMD17-02-1-0586

Tumor Secreted AMF: Causal Role in an Animal Model of Cachexia

INTRODUCTION:

Overview: This is the final report, although a crucial animal experiment has not yet been completed – for which we have requested (October 2005) a 6-month no-cost extension. An addendum summarizing these final results will be submitted in Spring 2006. **Summary**: Our original goal was to test critical whether AMF/PGI protein could cause cachexia. This has successfully accomplished. Additional goals were to establish an animal model for testing of the recombinant protein and to prepare various forms of purified recombinant AMF/PGI protein, mouse and human, for testing in the mouse model. These goals have also been accomplished. The practical combination of these two parts, however, was not successful, and we conclude that administration of the recombinant protein to mice is useful only as a proof-of-principle. We have focused the final experiments on an alternate and more robust animal model in which an AMF/PGI protein-secreting cell line (CHO-1C6) causes cachexia in mice carrying intramuscular tumors. In the experiments underway during the requested 6-month no-cost extension, the mechanisms (local versus systemic effects and identification of target genes activated in cachectic skeletal muscle cells) of AMF/PGI protein-induced cachexia will be assessed.

Update on cancer cachexia (September 2005). The previous progress reports provided extensive updates. Since then, little additional progress has been made to identify biochemical mechanisms leading to muscle wasting and atrophy. There have been no substantive breakthroughs in development of new animal models of cachexia, identification of additional circulating factors responsible for the syndrome, or the development of novel patient treatments (Tisdale, 2005). One of the factors identified by Tisdale, a small sulfated polypeptide called proteolysis-inducing factor, PIF (Wyke & Tisdale, 2004), has been controversial, since another laboratory was unable to reproduce cachexia with the recombinant human factor (Monitto et al 2004).

Biochemical mechanisms of cachexia: It appears that muscle wasting involves paracrine factors in the microenvironment surrounding muscle, which regulate its overall activity, in particular insulin-like growth factor-1 (Glass, 2003) and the TGF-beta superfamily member myostatin, also known as GFD-8, which is a negative regulator of muscle mass. (Roth & Walsh, 2004). IGF-1 appears to stimulate the PI3 kinase/Akt signaling and Foxo transcription factor pathways (Lee et al, 2004; Stitt et al, 2004; Sandri et al, 2004). The same pathways are activated during muscle wasting in response to multiple causes; so these are likely also to be activated during cancer-induced muscle wasting (Lecker et al, 2004). Myosin heavy chain is a major substrate for degradation in cachectic skeletal muscle (Acharyya et al, 2004).

Role of the proteasome. Skeletal muscle proteolysis in cachexia is probably due to increased activity through the proteasomal pathway, rather than via lysosomes or soluble sarcoplasmic proteases. Certain cancer treatments can either enhance or inhibit this muscle degradation pathway. Thus, cancer chemotherapy may alter cachexia in patients. Omega-3 fatty acids and other eicosanoids can regulate the activity of the proteasome, providing a biochemical rationale for the dietary treatment of cancer cachexia. It is not yet clear that activation of proteasomal degradation is the central or the only pathway for muscle wasting in cancer cachexia (Glass, 2003; Lecker, 2003), but this seems increasingly likely and may involve specific activation in muscle cells of the specific ubiquitin E3 ligases, atrogin and MuRF1 (Dehoux et al, 2004; Sachek et al, 2004) . This activation may involve the NFkappaB transcriptional pathway (Wyke & Tisdale, 2005).

This report summarizes our successful progress to establish that autocrine motility factor/phosphoglucose isomerase is a systemic cachectic factor. It has been known for 50 years to be elevated in patients with advanced, metastatic breast cancer, and we previously observed that it was also elevated in the circulation of nude mice that were cachectic due to advanced breast cancer metastases restricted to bone.

BODY OF FINAL REPORT

Timetable: The award of this grant was made just as the Principal Investigator was moving from the University of Texas to the University of Virginia. Initial work was commenced upon arrival in Charlottesville Virginia in October 2002. A new research associate was recruited to work on this project, Ms. Lisa Wessner, who has learned all of the techniques specific to the project, which has now been active since January 1 of 2003.

Thus, this final report represents work carried out over the two year period from July 2003 through August 2005. The main animal experiments were substantially delayed by an outbreak of the lethal mouse pathogen *Burkholderia gladioli* (Foley et al, 2004).

The **revised application** contained **3 Specific Aims and 9 Tasks** in the final, Approved **Statement of Work:**(Revised 01/04/02 with original Aims 4 & 5 deleted):

Task 1 (Aim 1) Determine dose range and Alzet mini-pump size for administration of mAMF to achieve effective blood levels of ~10ng/mL. [28 mice] Year 01, months 1-6.

Task 2 (Aim 1) Demonstrate cachexia with mouse AMF infused into mice, compared to controls and to mice bearing CHO-K1 [mAMF-] and CHO-1C6 [mAMF+] IM tumors. [56 mice] Year 01, months 7-12.

Task 3 (Aim 1) Carry out routine pathology and histology of animals from Task 2. Year 01, months 10-12.

Task 4 (Aim 1) Determine host concentrations of 4 host cytokines in baseline and sacrifice blood samples of animals from task 2. Year 02, months 1-4.

Task 5 (Aim 2) Construct two mutants of mAMF [E357A and 4S/T-A] and sequence. Year 02, months 1-4.

Task 6 (Aim 2) Express and purify mutant mAMF proteins and determine Km and Vmax and binding to phosphocellulose. Year 02, months 5-8.

Task 7 (Aim 2) Carry out Task 2 & 3 protocols with wt & 2 mutant mAMFs by pump infusion. [24 mice] Year 01, months 9-2; Year 02, throughout.

Task 8 (Aim 3) Carry out Task 2 & 3 protocols with mouse versus 3 concentrations of human AMF. [40 mice] Year 02, months 1-12.

Task 9 (Aims 1-3) Analyze data, prepare and submit results for meeting presentations, progress reports, and peer-reviewed publication. Year 01-02, throughout.

Total mice requested = 148 [adult female Balb/c & Balb/c nudes, 32 mice were eliminated in deleted Aim 5]. All use of human cell lines and radioisotopes was eliminated with the deletion of Aims 4 & 5. Animal use has been reported separately to the DoD. Since the annual animal usage and progress reports cover different times, the most recent animal usage form did not include the mice that were used in Figures 1 and 2.

Progress:

Task 1 was completed in the first year.

Task 2 has been completed with a simplified delivery protocol. Animals were given the factor as sterile intraperitoneal (i.p.) injections of protein in PBS at 8 AM, noon, and 4 PM. Blood levels of PGI/AMF were measured at the 4PM time, and the animals were weighed. The injected mouse AMF/PGI was entirely cleared from the blood stream by 24 hours. The previous report includes figures showing a substantial animal experiment in which nude mice were injected 3 time per day for 3 days with 100 μl of sterile PBS containing purified mouse AMF/PGI or BSA (negative control) at either 150 or 500 μg of protein per dose. The data demonstrate that 150 μg doses were ineffective to cause weight loss, while 500 μg doses caused a statistically highly significant cachexia by 24 hours after the initial injection, which appeared to persist for 24hrs after the cessation of injection following three days of treatment.

  Mouse AMF/PGI for these experiments was prepared in E coli and purified by NiNTA chromatography using the 6xHis C-terminal extension. As detailed in the first progress report, these recombinant protein preparations were carefully analyzed for contamination with bacterial endotoxin and determined to be free of this material to well below a level that would cause inflammation in the animals. The laboratory animal personnel report that the AMF/PGI treatments did not cause any apparent pain or discomfort to the mice.

  Task 3 was carried out and no changes in the routine histology of the mice was observed. Similarly, for **task 4**, no changes in plasma concentrations of interleukins 1, 6 or TNFalpha were observed, while changes in a fourth known cachectic mediator, parathryroid hormone-related protein, PTHrP, were not detectable.

Tasks 5 and 6 have been completed. The catalytically inactive mutant E357A was been constructed, expressed and purified. In collaboration with Dr Christopher Davis at the University of South Carolina, the x-ray crystal structure of this mutant was been determined. The data were originally included in the manuscript published in J Mol Biol, as Solomons et al (below under Research Accomplishments), but the data on the mutant were removed to keep the paper within acceptable page limits and will be submitted later. The role of isomerase (PGI) activity in relation to autocrine motility factor (AMF) activity appears no longer to be controversial in the field. We (Davies et al, 2003) and others (Arsenieva & Jefferey, 2002), have shown that ligand binding to mammalian PGIs results in only very small conformational changes in the surface of the protein away from the active site (where binding to the AMF receptor almost certainly takes place). These conformational changes are almost certainly insufficient to have any effect on a receptor-mediated cytokine-like action of the protein. We believed that mouse E357A AMF/PGI would be active in the cachexia assay. This was proposed in Task 7, which was carried out during 2005. This experiment was entirely unsuccessful. During the course of our work with the model, we made successive improvements in the animal husbandry; such that repetitive anesthesias and retro-orbital blooding drawing were eliminated. These changes resulted in a substantial reduction in physiological stresses on the mice to the point that they no longer lost body mass in response to repeated injections of the AMF/PGI protein at does that were previously cachectic. Since these does were already high, we concluded that Task 7, although completed, was an experimental failure that was impractical to pursue further. Since Task 7 failed to produce cachectic mice, we had no specimens on which to complete Task 8.

We are now carrying out Task 8, the cachexia model, with mice carrying the CHO-1C6 tumor cell line or its control CHO-K1. The 1C6 cells secrete mouse AMF/PGI. Use of this line makes it impossible for us to compare in vivo the species-specific actions of mouse versus human AMF/PGI proteins, since a human PGI-secreting cell line is not available. Details of this model are provided in the appended pdf documents from the P.I.'s presentations at the 2005 DoD-sponsored Era of Hope meeting. Mice with tumors and cachexia will be sacrificed and tumor, adjacent muscle, and matched contralateral muscles harvested. Muscle RNAs will be assayed by real-time PCR with mouse-specific primer pairs for a series of markers: IGF-1, myostatin, atrogin, MuRF-1, myosin heavy chain, and Foxo 3. We will also prepare muscle homogenates from treated and control mice and assay them for proteasomal activity (Reinheckel et al, 2000) as was shown in figure 3 of the previous report.

Task 9. We have published several papers on the AMF/PGI and several reviews on bone metastases including discussions of metastasis-associated cachexia. Most recently we have published an animal model of metastasis (albeit of bladder cancer) to the lung, in which metastasis is suppressed by the signaling regulator Rho GDI2 (Titus et al, 2005). We found that the two major targets of metastasis suppression by Rho GDI2 were endothelin-1 and neuromedin U. The focus of the Titus et al paper was the role of endothelin in metastases, which were effectively decreased by an orally active endothelin antagonist already in clinical trials. We are currently pursuing the role of neuromedin U, particularly since this molecule can cause weight loss in overexpressing animals (Kowalski et al, 2005) and is likely to be an important mediator of cancer anorexia and perhaps cachexia. We have preliminary data that these same gene regulatory pathways and responses also occur in breast cancer cell lines. We have not yet

published the details of our two animal models in which AMF/PGI causes cachexia. These two papers would be greatly strengthened by a demonstration of molecular effects on muscle protein degradation in vivo. The final animal experiment should provide these missing data and will permit us to submit these final publications in 2006.

SUPPORTING DATA

This document is accompanied by three appendices, two of which are pdfs of the presentations (PowerePoint and Poster) made by the P.I. These two presentation pdfs provide extensive illustrations of the points described above.

KEY RESEARCH ACCOMPLISHMENTS:

1) Purification of recombinant AMF/PGI with low endotoxin content finalized.

2) Animal model of direct i.p. injection of mouse AMF/PGI used to cause statistically significant (4%) weight loss in 24hrs compared to equivalent control protein treatment. Partial dose-response established between 450 (ineffective) and 1500 (effective) µg/mouse/day. Data are shown in **Figures 1** and **2**, below.

3) Human AMF/PGI prepared as in 1)

4) Crystal structures of mouse and human AMF/PGI, used in all of the work for this proposal, solved and published in a pair of papers in the Journal of Molecular Biology

5) In vitro assay for proteasome activation in mouse C2C12 myoblasts established and responses to mouse AMF/PGI demonstrated. Data are shown in **Figure 3**, below

6) Animal model of cachexia with direct injection of AMF/PGI protein validated and limitations defined. Data are shown in **Figures 1** and **2**, below

7) Animal model of cachexia due to implantation of AMF/PGI protein-secreting tumor characterized.

8) Animal model of lung metastasis with a cancer cell line overexpressing the anorexigenic peptide neuromedin U published (Titus et al, 2005). Neuromedin U causes weight loss in mice (Kowalski et al, 2005) and we are pursuing studies with this new animal model.

REPORTABLE OUTCOMES (previous two years):

Twelve manuscripts published, which include reviews of the contributions of bone metastases to cancer cachexia, while two papers (*'ed) derive directly form the work carried out for this proposal:

Chirgwin JM, Guise TA. Interactions between tumor and bone alter the phenotypes of both. J Musculoskel Neuronal Interact, 3:278-281, 2003.

Chirgwin JM, Guise TA. Molecular mechanisms of cancer metastases to bone. Curr Opin Orthop, 14:317-321, 2003.

Guise TA , Chirgwin JM. Role of TGFβ in osteolytic bone metastases. Clin Orthop, 415:S32-38, 2003.

Chirgwin JM, Guise TA. Cancer metastasis to bone. Science & Medicine, 9:140-151, 2003.

Chirgwin JM, Mohammad KS, Guise TA. Tumor-bone cellular interactions in skeletal metastases. J Musculoskeletal Neuronal Interact 4:308-318, 2004. Copy previously provided.

*Graham Solomons JTG, Burns S, Wessner L, Krishnamurthy N, Zimmerly E, Swan MK, Krings S, Muirhead H, Chirgwin J, Davies C. The crystal structure of mouse phosphoglucose isomerase at 1.6 Å resolution and its complex with glucose 6-phosphate reveals the mechanism of sugar ring opening. J Mol Biol. 342:847-860, 2004. Copy previously provided.

Guise TA, Chirgwin JM. Biology of bone metastases. Chapter in Diseases of the Breast, 3rd edition. Harris, Lippman, Morrow, and Osborne (eds). Lippincott Williams & Wilkins, pp 1285-1296, 2004.

GuiseTA, KozlowWM, Heras-Herzig A, Padalecki SS, Yin JJ, Chirgwin JM. Molecular mechanisms of breast cancer metastases to bone. Clin Breast Cancer 5 Suppl 2:S46-53, 2005.

*Titus B, Frierson HF Jr, Conaway M, Ching K, Guise T, Chirgwin J, Hampton G, Theodorescu D. Endothelin axis is a target of the lung metastasis suppressor gene RhoGDI2. Cancer Res 65:7320-7327, 2005.

Clines GR, Chirgwin JM, Guise TA. Skeletal complications of malignancy: central role of the osteoclast. Bone Resorption, Topics in Bone Cell Biology, vol 2. Bronner F, Farach-Carson MC, Rubin J, eds. Springer, London; pp 151-174, 2005

Zudaire E, Martinez A, Garayoa M, Pio R, Kaur G, Woolhiser MR. Metcalfe DD, Hook WA, Siraganian RP, Guise TA, Chirgwin JM, Cuttitta F. Adrenomedullin is a cross-talk molecule that regulates tumor and mast cell function during human carcinogenesis. Am J Pathol, accepted for publication, 2005.

Bendre MS, Aaron G. Margulies AG, Walser B, Akel NS, Bhattacharrya S, Skinner RA, Swain F, Ramani V, Mohammad KS, Wessner LL, Martinez A, Guise TA, Chirgwin JM, Gaddy D, Suva LJ. Tumor Cells can Stimulate Osteoclast Formation and Osteolysis in the Absence of RANKL. Cancer Res, accepted for publication, 9/15/05

CONCLUSIONS

Purified mouse autocrine motility factor/phosphoglucose isomerase causes statistically weight loss (cachexia) after 3 days of 3X daily intraperitoneal injection at a dose of 0.5 mg/injection, which was accompanied by significant increases in serum concentrations of the factor. This is a simpler animal model than originally proposed. Thus, the main hypothesis of the original proposal has been validated. The injection model, while establishing that AMF/PGI protein is a causal agent for cachexia, has not been practical as animal model for the detailed structure/function analysis of the factor. It has also not been practical as a model for the elucidation of the molecular mechanisms by which the factor is presumed to activating skeletal muscle proteolysis. We are completing the project with an additional experiment using a tumor bearing animal model that in our hands reliably causes cachexia. We will use this to identify genes activated in skeletal muscle in response to secreted AMF/PGI protein and to test whether such gene activation occurs locally adjacent to tumors or is systemic.

Structures of the AMF/PGI proteins, including mouse and human proteins and the enzyme complexed with inhibitor have been solved by x-ray crystallography and published or accepted for publication. Mutant forms of the protein have been prepared. Experiments have been successfully completed to improve the purity of the recombinant protein and to characterize the effects of the factor on both intact animals and on a mouse muscle cell line in vitro. The structural data are now complete for understanding the species-specific effects and their structural bases.

REFERENCES ADDED FOR 2005:

Kowalski TJ, Spar BD, Markowitz L, Maguire M, Golovko A, Yang S, Farley C, Cook JA, Tetzloff G, Hoos L, Del Vecchio RA, Kazdoba TM, McCool MF, Hwa JJ, Hyde LA, Davis H, Vassileva G, Hedrick JA, Gustafson EL (2005). Transgenic overexpression of neuromedin U promotes leanness and hypophagia in mice. J Endocrinol 185:151-64.

Monitto CL, Dong SM, Jen J, Sidransky D (2004). Characterization of a human homologue of proteolysis-inducing factor and its role in cancer cachexia. Clin Cancer Res 10:5862-9.

Tisdale MJ (2005). Molecular pathways leading to cancer cachexia. Physiology 20:340-8

Wyke SM, Tisdale MJ (2005). NF-kappaB mediates proteolysis-inducing factor induced protein degradation and expression of the ubiquitin-proteasome system in skeletal muscle. Br J Cancer 92:711-21.

Additional references are in a comprehensive list provided in the second annual progress report.

10

FIGURES and LEGENDS
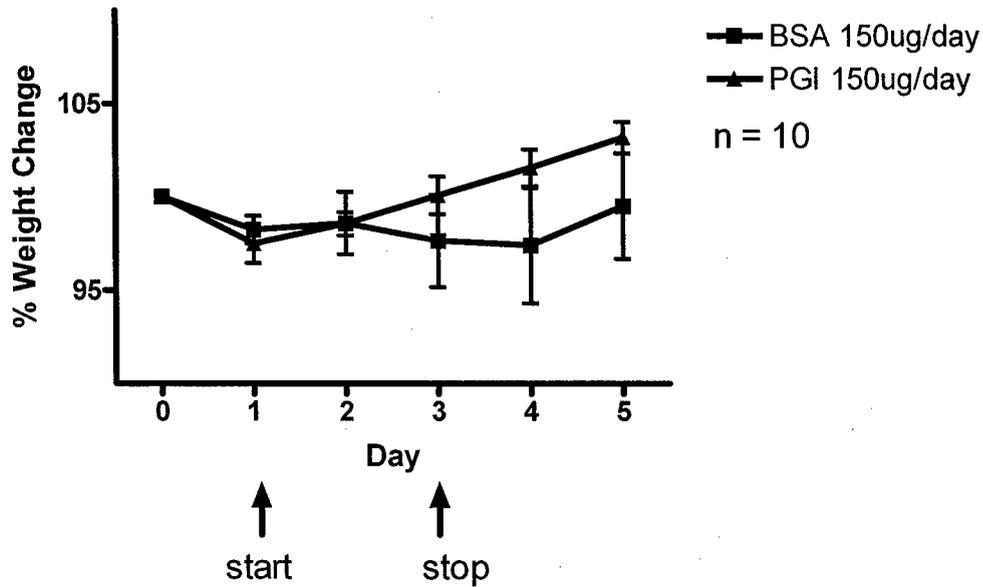
Figure 1. In the experimental protocol, Balb/C nu/nu mice, approximately 25g in weight, were given the factor as sterile intraperitoneal (i.p.) injections of protein in PBS at 8 AM, noon, and 4 PM. Blood levels of PGI/AMF were measured at the 4 PM time. Animals were weighed at the same time. Animals were injected on days 1, 2, and 3 and allowed to recover for two more days. Injections were of 150 μg purified mouse AMF/PGI or BSA (Sigma) in 100 μl of sterile PBS. The curves are not statistically significant (p > 0.05) between the two groups by 2-way ANOVA, with n = 10 mice/group.
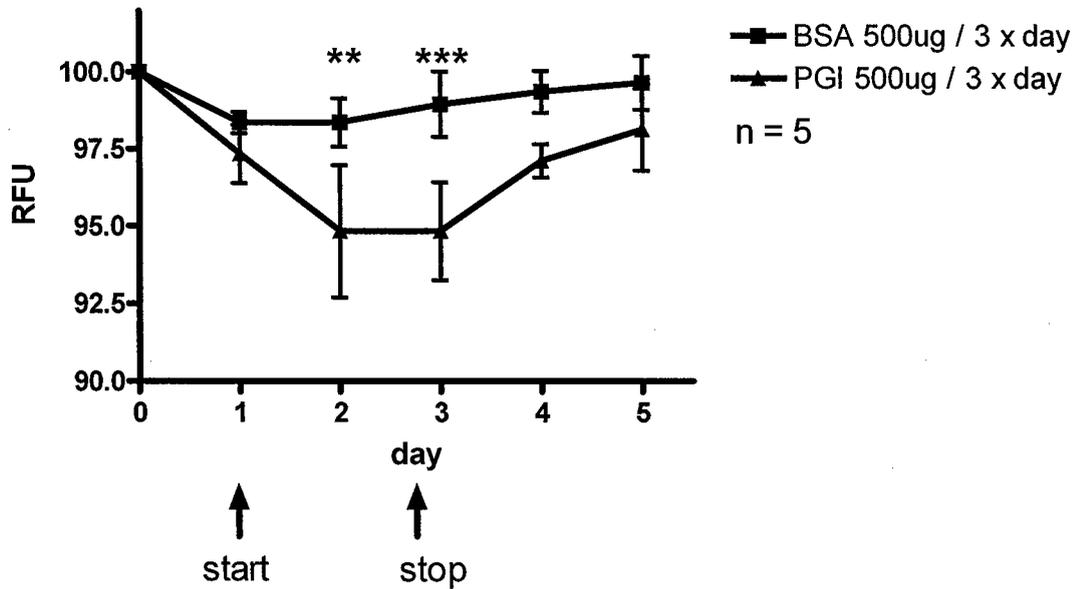
Figure 2. Protocol was similar to that described under Figure 1, except that the injections were of 500 μg aliquots of the proteins, in the same volume of PBS as before. There were n = 5 mice per group. The curves were analyzed for statistical significance by 2-way ANOVA, and p < 0.0001. Significance of differences between the two curves at each time point were calculated by comparing replicates of the means by row with Bonferroni's post-test using the statistics programs in GraphPad Prizm. ** p < 0.01; *** p < 0.001. Other points n.s. (p > 0.05).

20S Proteasome Assay
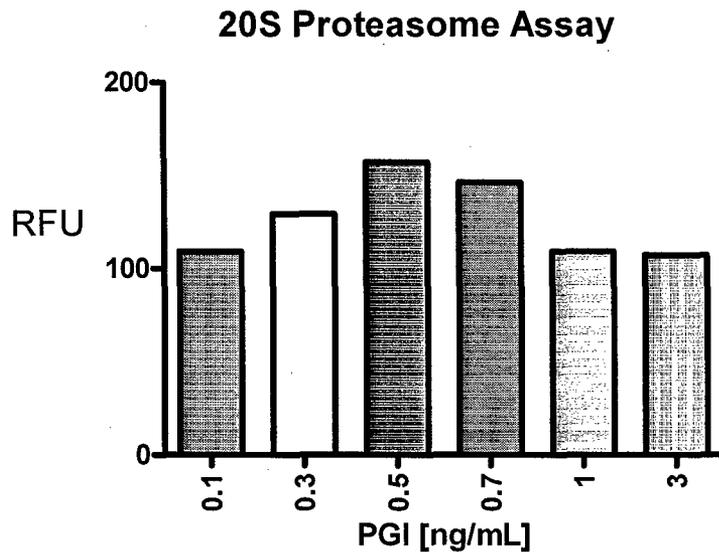
Figure 3. C2C12 mouse myoblasts (ATCC) were grown to confluence, changed to serum-free medium for 24 hrs, and then treated for 24 hrs with the indicated concentrations of recombinant mouse PGI/AMF. These cells are routinely studied for the induction of proteasomal activity (Coulombe et al, 2003: You et al, 2003), as well as being used for models of skeletal muscle cell function and differentiation (Yamaguchi, 1995). Cells were harvested, lysed by three cycles of freeze/thawing, homogenized, and centrifuged for 10 minutes at 14,000xg and 4°C to sediment debris. The supernatants were adjusted to a constant protein concentration of 50 μg/ml and assayed for 20S proteasomal chymotryptic activity (Reinheckel et al, 2000) with the fluorogenic substrate, succinyl-LLVY-AMC (Calbiochem) for 1 hr at 37°C. Control values not shown, since these continue to be highly variable, and we have not yet succeeded in establishing a reproducible negative control for the assay. Differentiation of the myoblasts into myotubes, by culturing in the presence of horse serum, may resolve this problem (experiments underway).
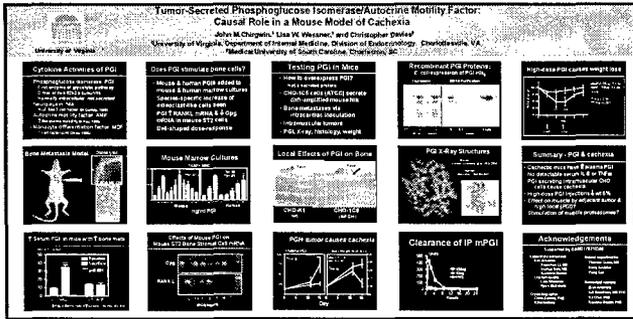
Tumor-Secreted Phosphoglucose Isomerase/Autocrine Motility Factor: Causal Role in a Mouse Model of Cachexia

Tumor-secreted Phosphoglucose Isomerase/Autocrine Motility Factor Causal role in a mouse model of cachexia

9 June 2005

John Chirgwin, PhD
Department of Medicine
University of Virginia

http://titan.med.virginia.edu/AurbachLabs/

Cytokine Activities of PGI

- **Phosphoglucose isomerase, PGI**
  - First enzyme of glycolytic pathway
  - Dimer of two 62kDa subunits
  - *Normally intracellular, not secreted*

- **Neuroleukin, Nlk**
  - NGF-like T cell factor (M Gurney, 1988)

- **Autocrine motility factor, AMF**
  - ↑ Melanoma mobility (A Raz, 1996)

- **Monocyte differentiation factor, MDF**
  - T cell factor (J-W Chiao, 1996)

Bone Metastasis Model

Osteolytic

MDA-MB-231
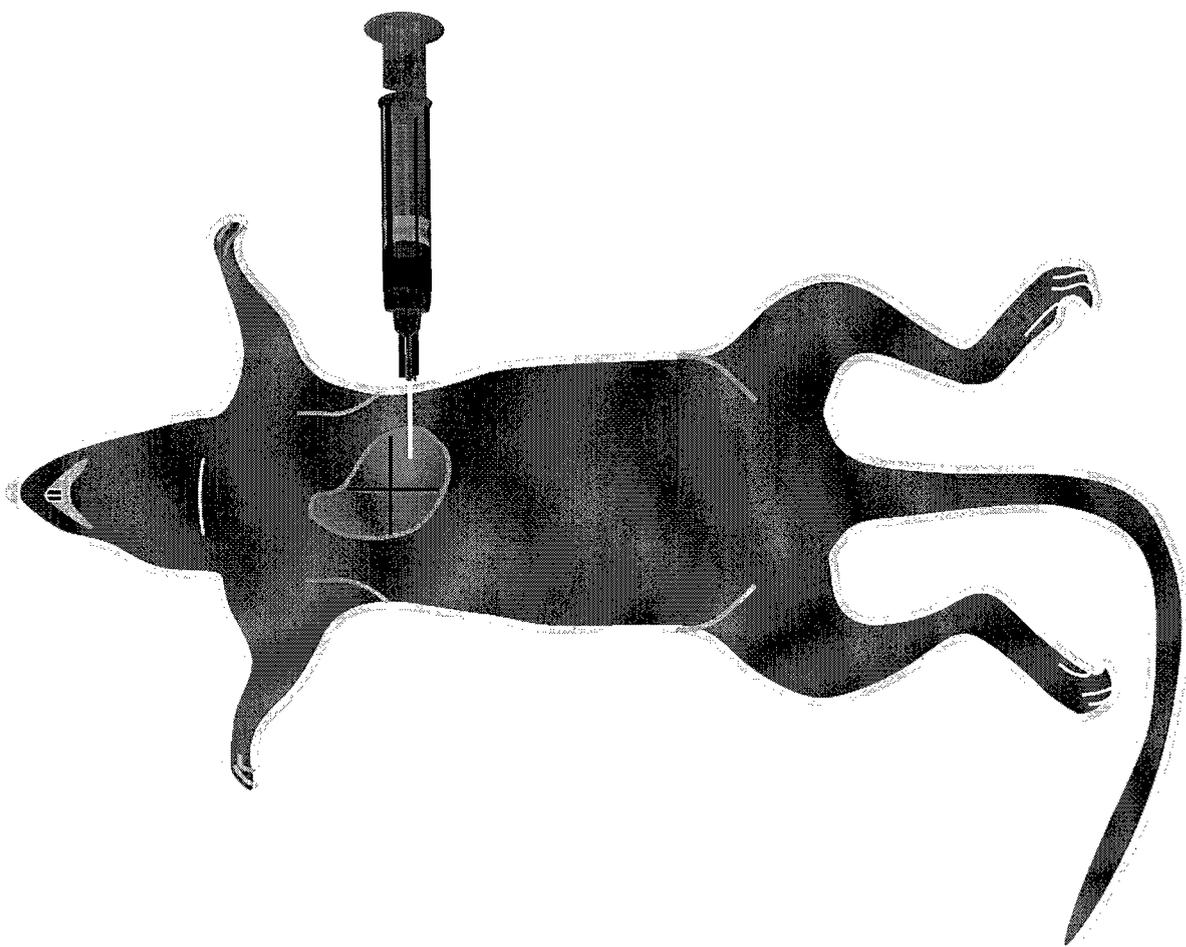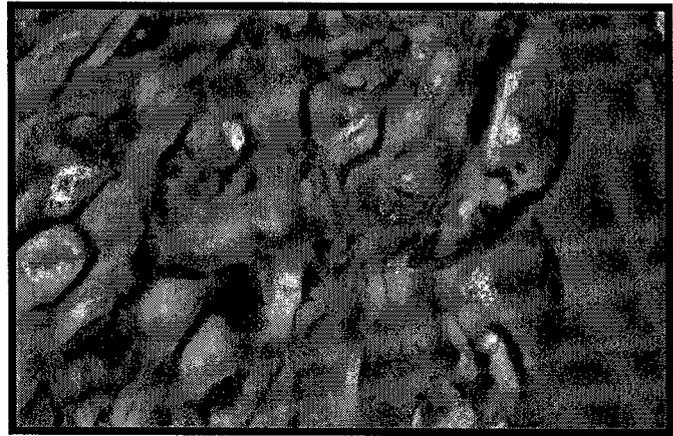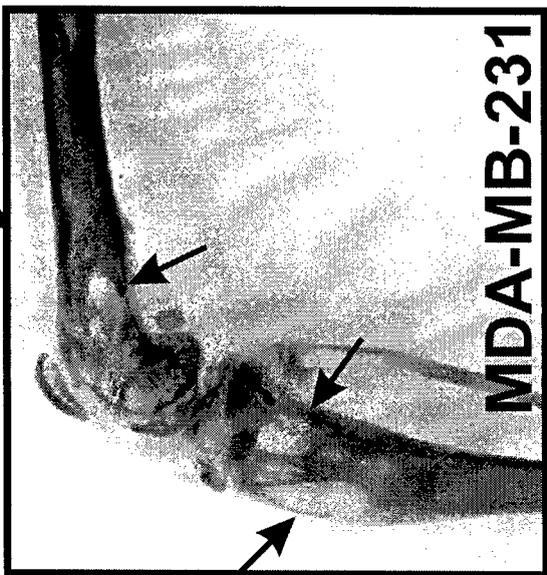
↑ Serum PGI in mice with ↑ ↑ bone mets

Units of PGI activity

Legend:
- Baseline
- Sacrifice

*** p<0.001

pcDNA3   TβRIIΔcyt
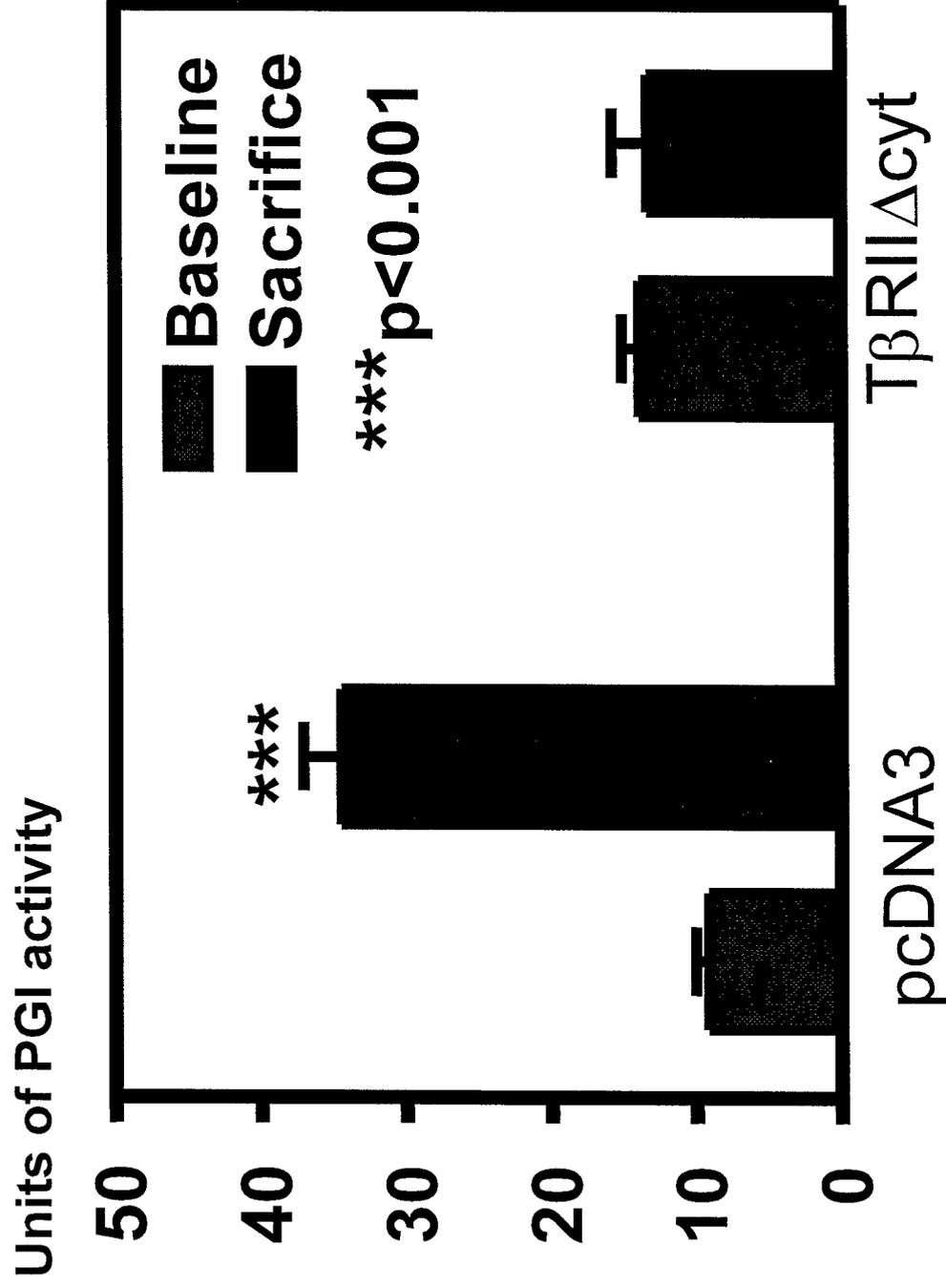
- TβRIIΔcyt ↓ bone mets & ↑ survival – Yin et al, JCI, 1999

Serum PGI in Breast Cancer Patient
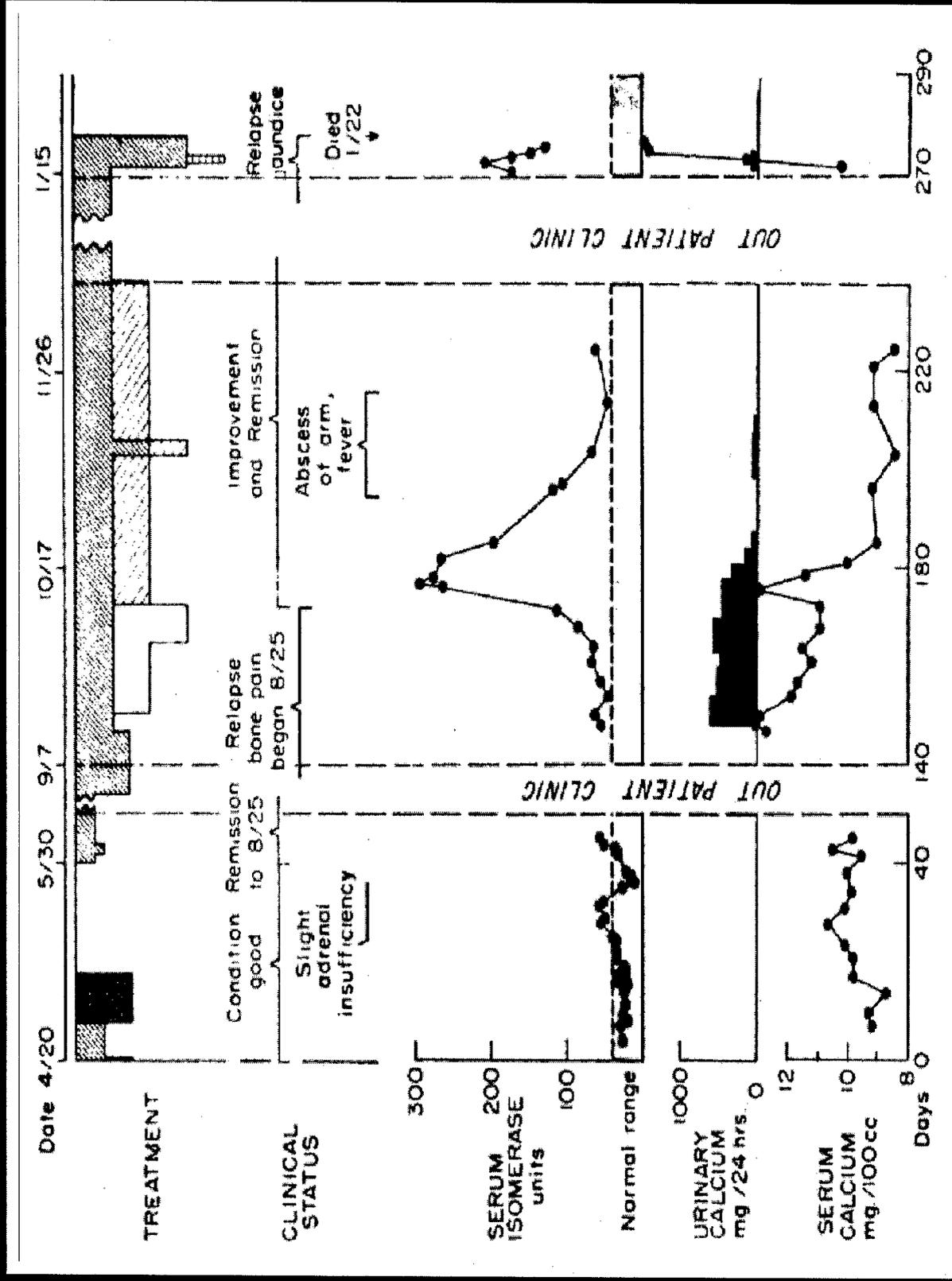
- O. Bodansky, Cancer (1954)

Does PGI stimulate bone cells?

- Mouse & human PGIs added to mouse & human marrow cultures

- Species-specific increase of osteoclast-like cells seen

- PGI $\uparrow$ RANKL mRNA & $\downarrow$ Opg mRNA in mouse ST2 cells

- Bell-shaped dose-response

Mouse Marrow Cultures

TRAP+ MNC

*p<.05
**p<.01

***p<.001

200

100

0

Human

Mouse

ng/ml PGI

C   vitD3 PTHrP 0.01   0.1   1   10   100

C VitD PTHrP 0.01 0.05 0.1 0.5 1 5 10 100
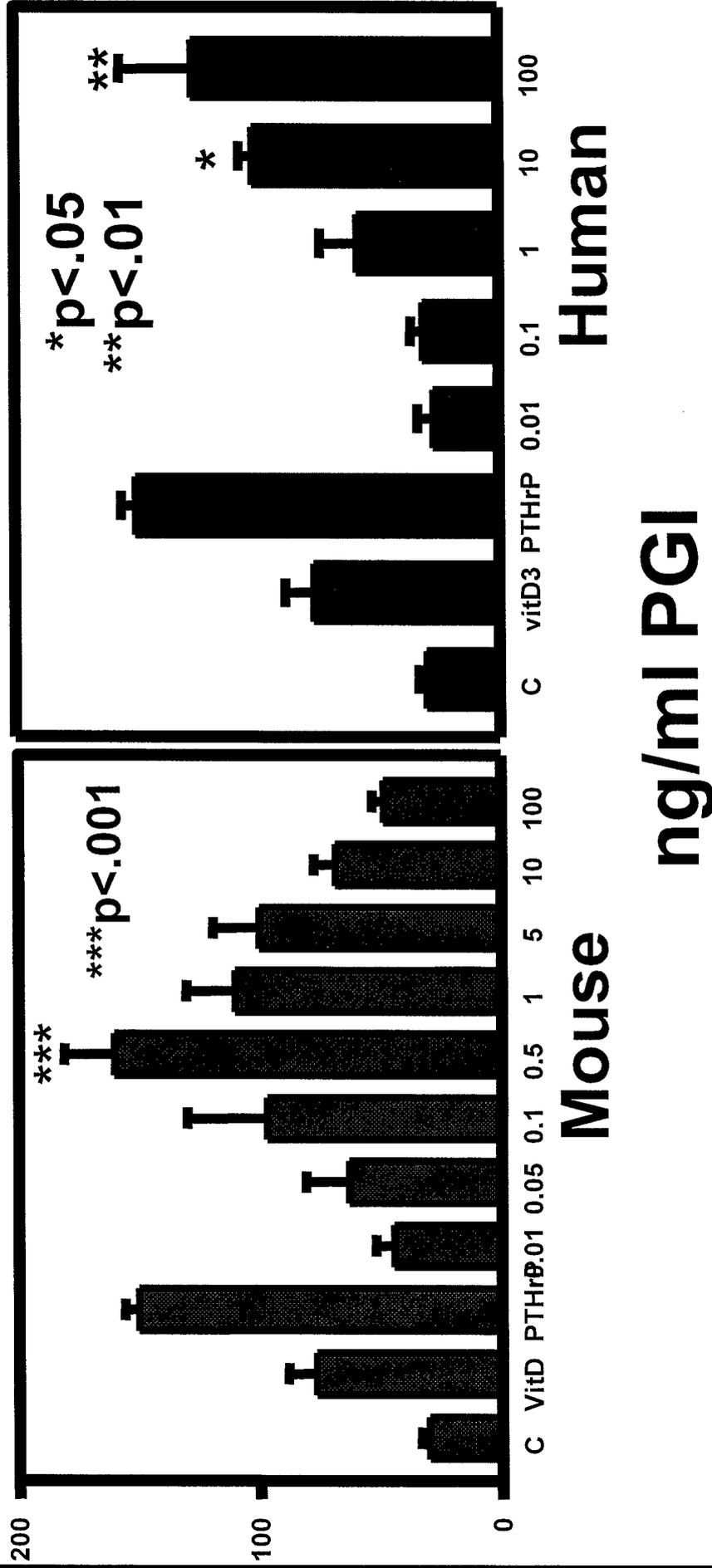
Human Marrow Cultures

23C6+ MNC
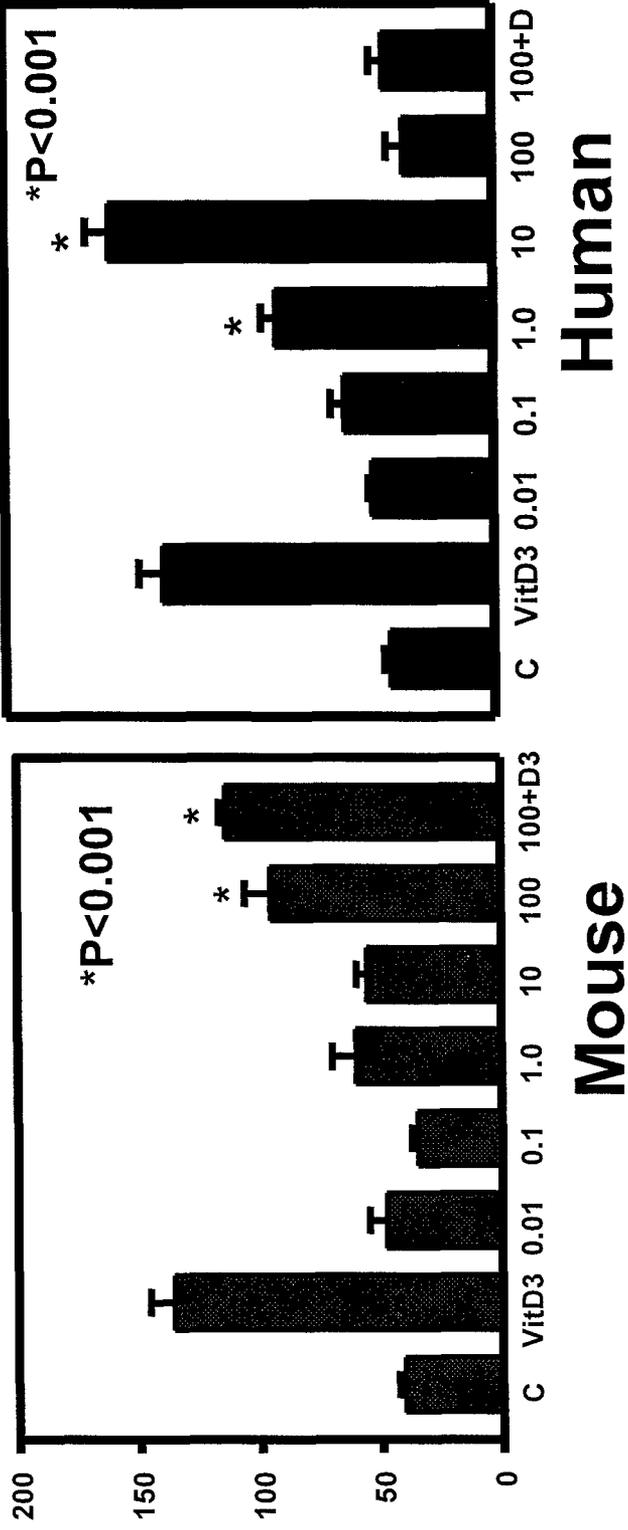
ng/ml PGI

Effects of Mouse PGI on Mouse ST2 Bone Stromal Cell mRNA
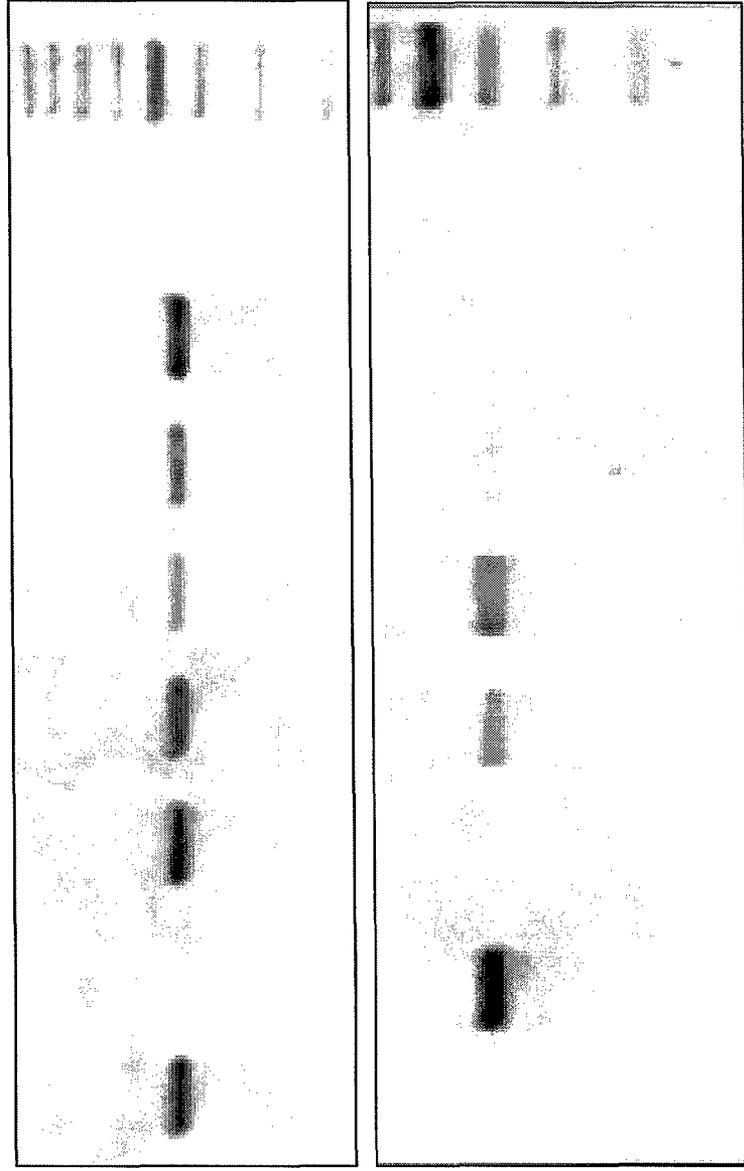
Opg

RANK L

C   D$_3$ 0.5   1   2   5   10

[PGI] (ng/ml)

Testing PGI in Mice

- How to overexpress PGI?
  - Not a secreted protein
- CHO-1C6 cells (ATCC) secrete dhfr-amplified mouse NIk
- Bone metastases via intracardiac inoculation
- Intramuscular tumors
- PGI, X-ray, histology, weight

PGI Secretion In Vitro
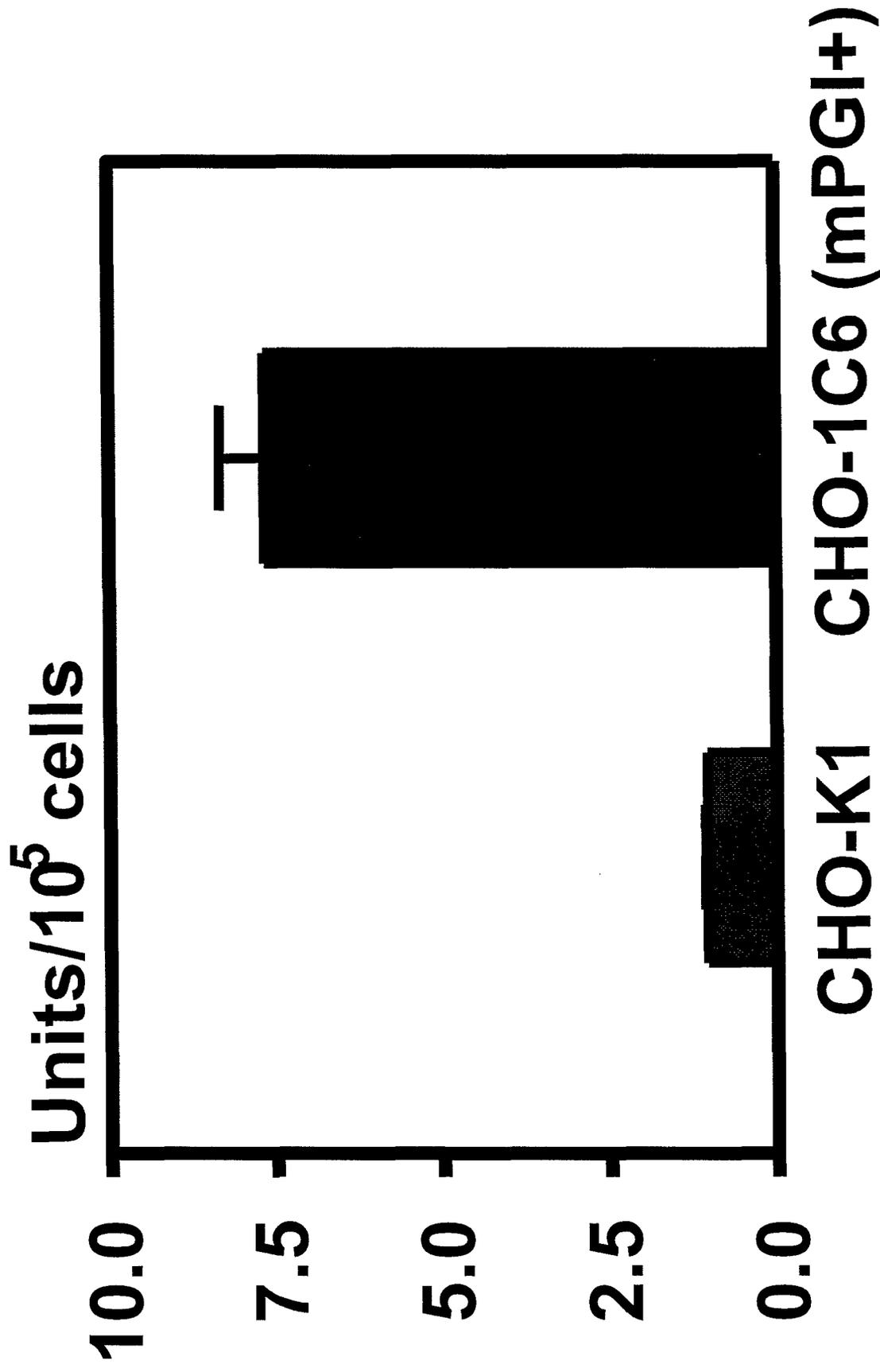
IM tumors affect bone

CHO-1C6
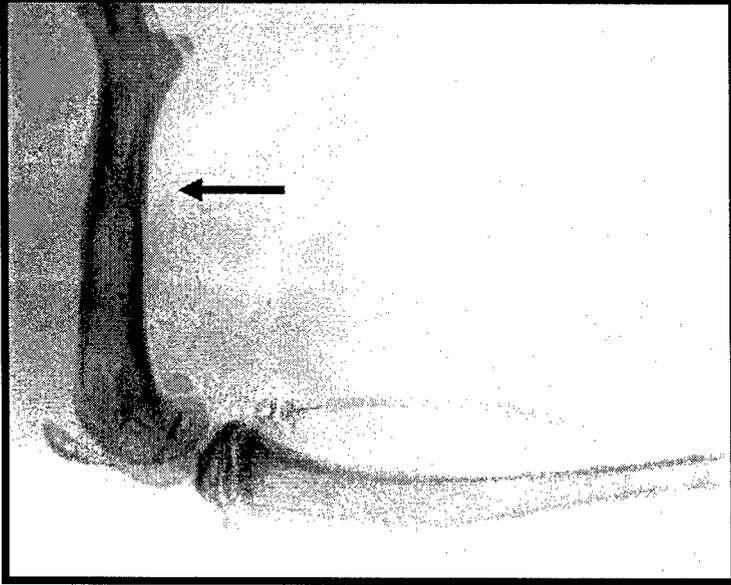
CHO-1C6

CHO-K1

PGI and Bone Metastasis

No Tumor
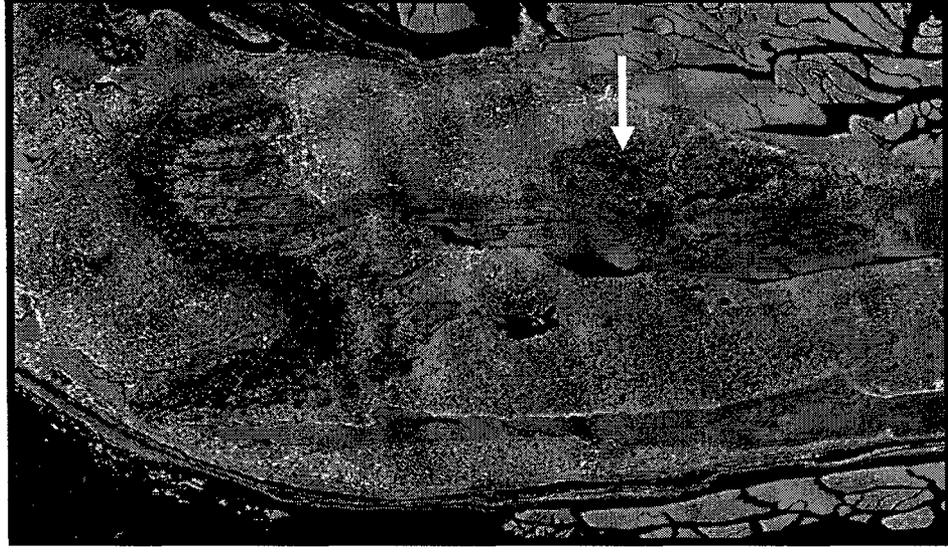
CHO-1C6
(mPGI+)
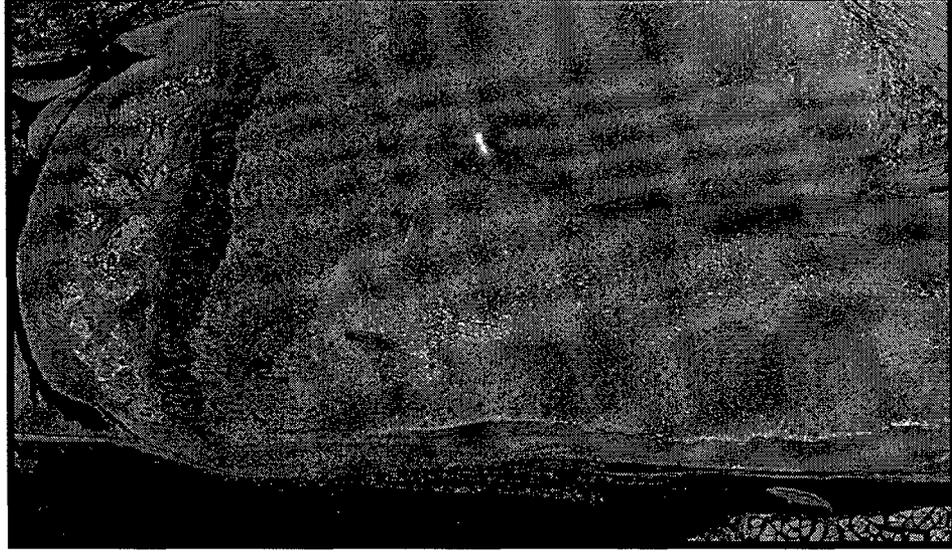
CHO-K1
(wt)

Local Effects of PGI on Bone
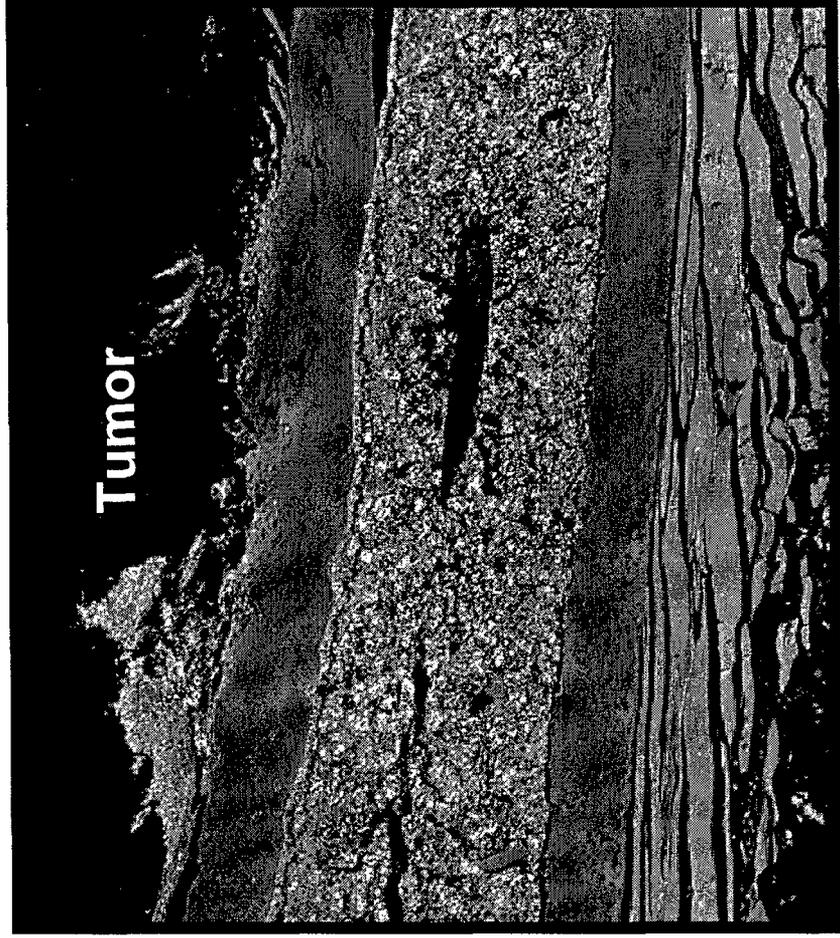
CHO-1C6
(mPGI+)

CHO-K1
(wt)
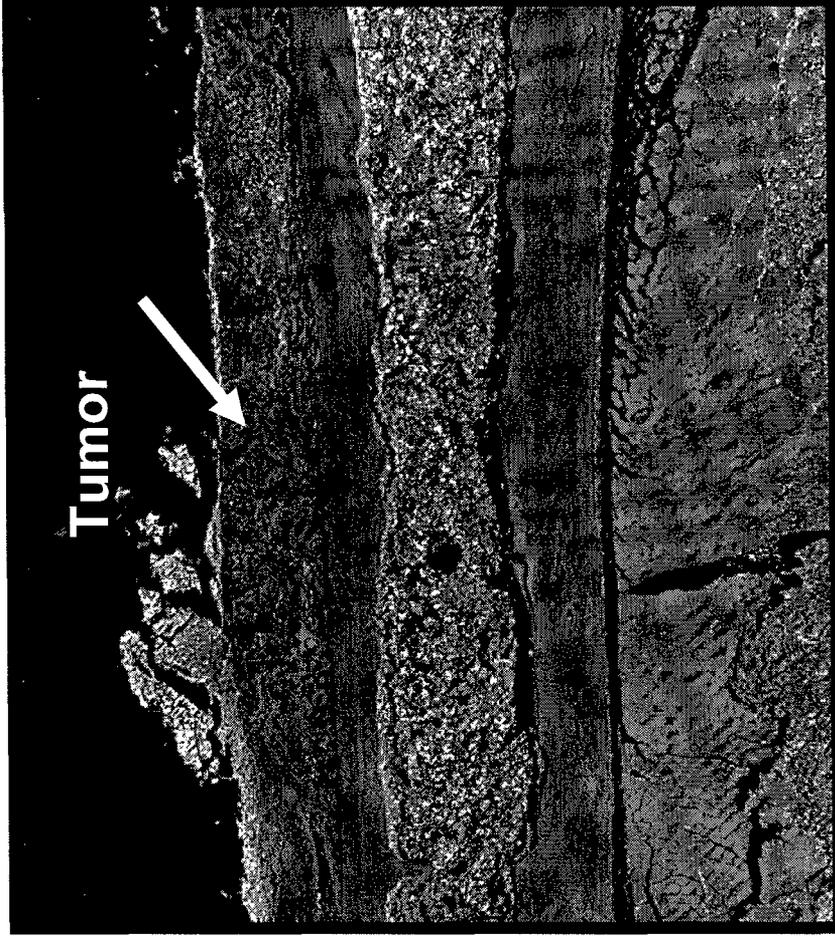
PGI+ tumor causes cachexia

Body Weight (% of day 0)

CHO-1C6
CHO-K1

*P<0.05

Plasma PGI

*P<0.05

Day
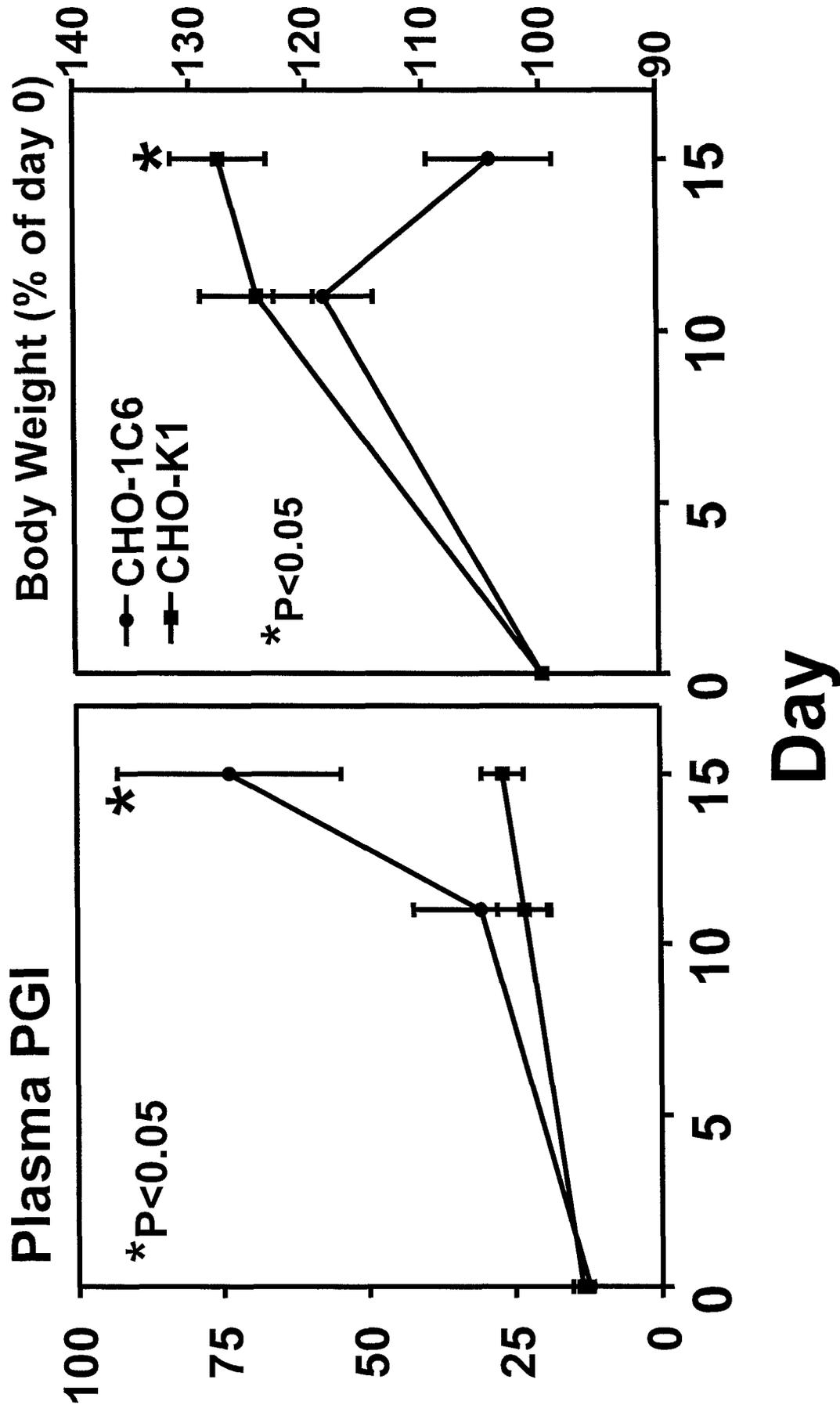
Recombinant PGI Proteins:
E. coli expression of PGI-His$_6$
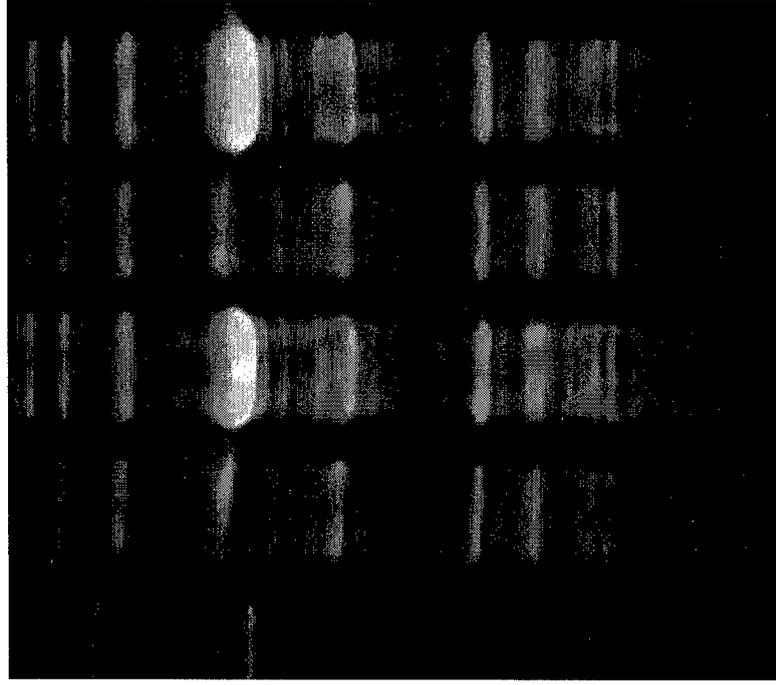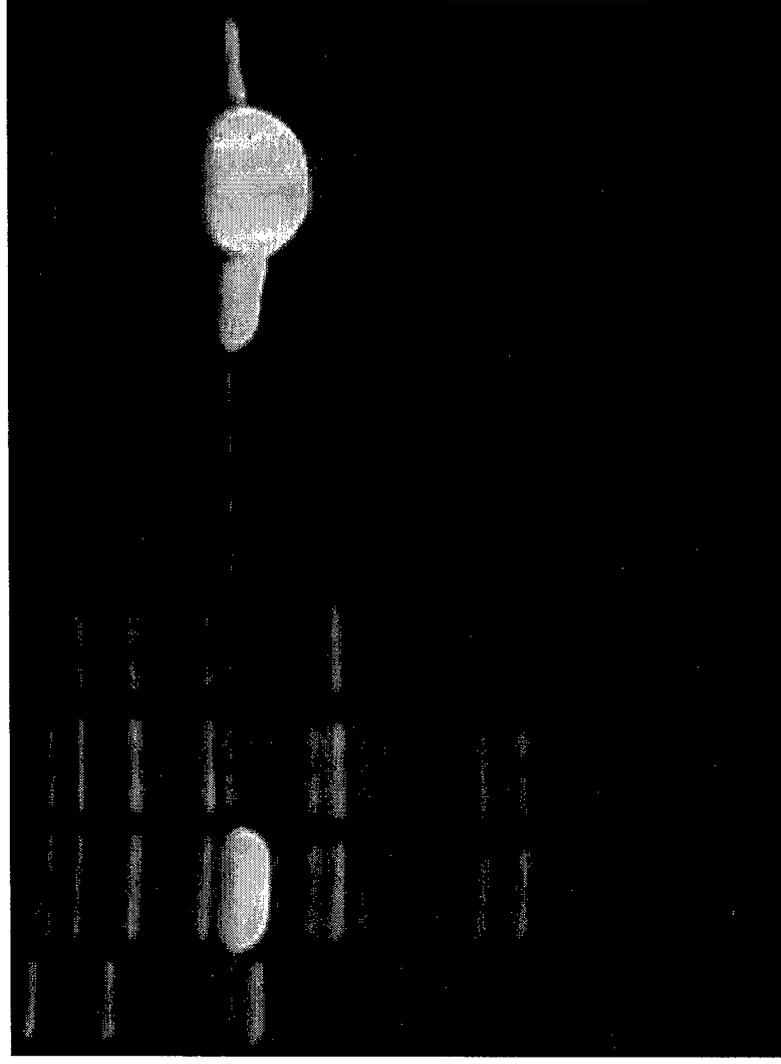
PGI X-Ray Structures

Mouse
-Graham Solomons et al, JMB 2004

Human
- Read et al, J Mol Bio 2001
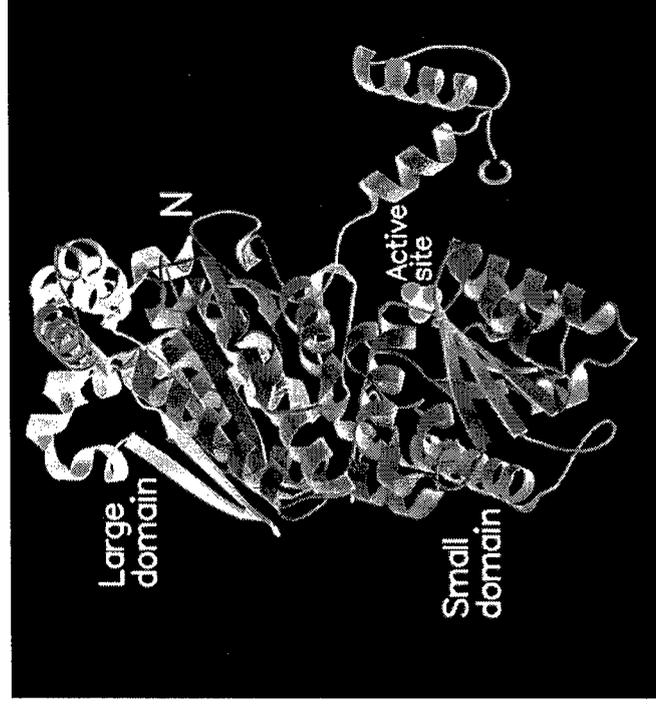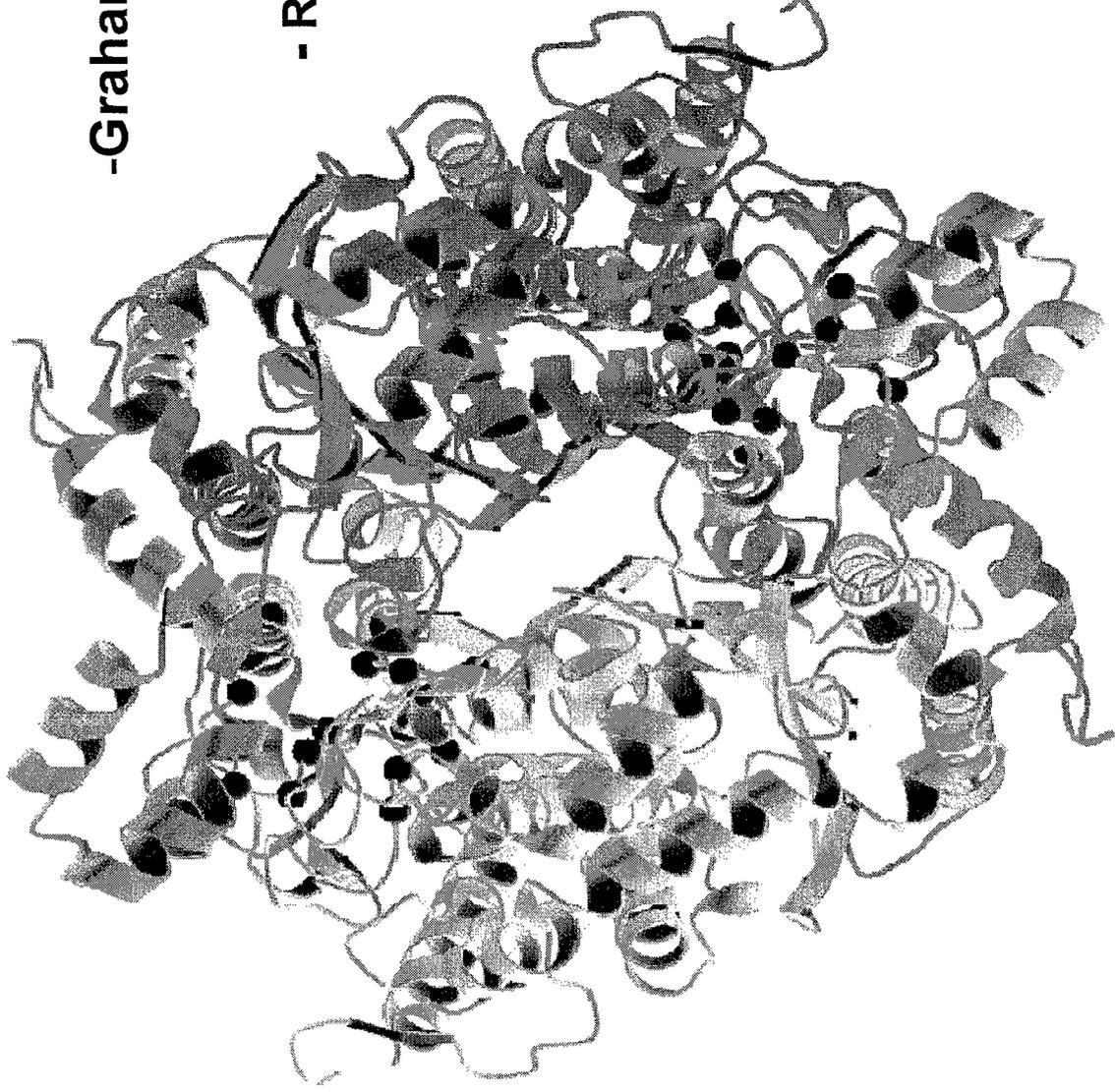
Clearance of IP mPGI
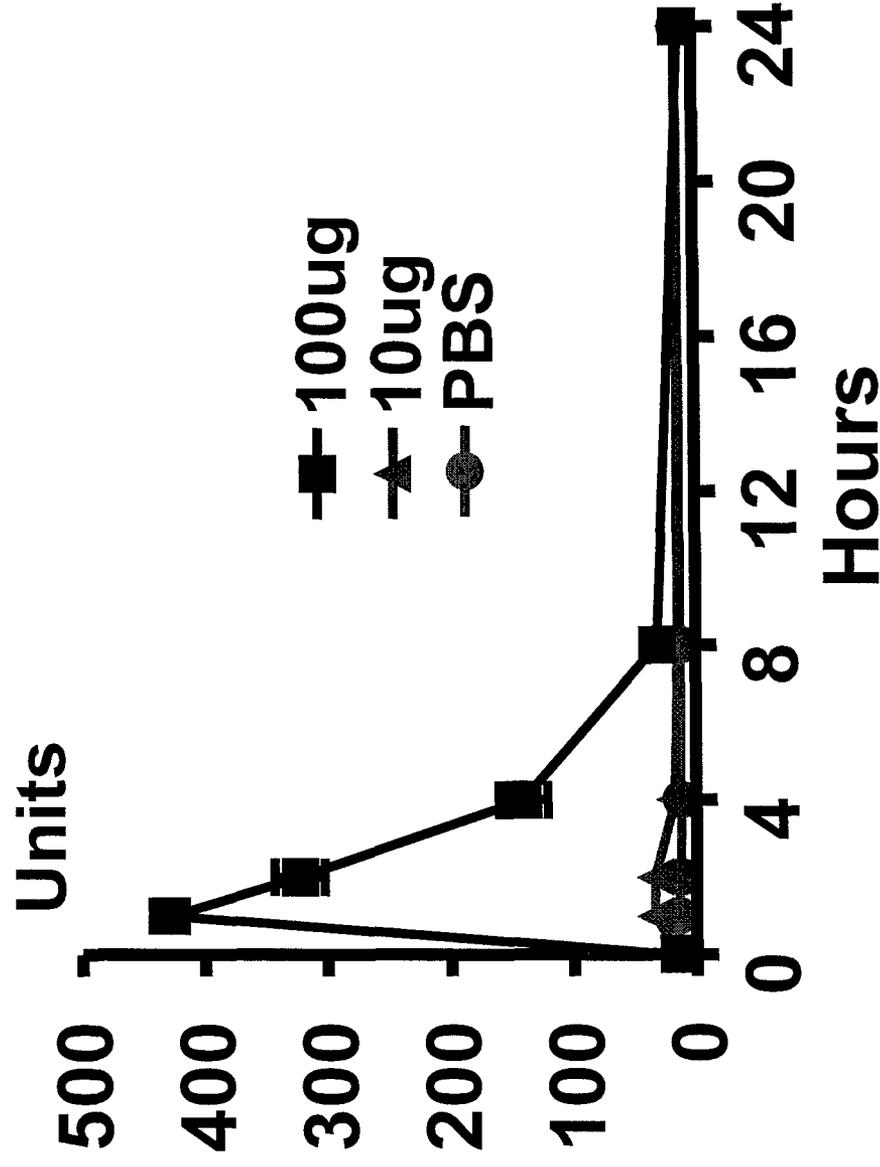
High-dose PGI causes weight loss
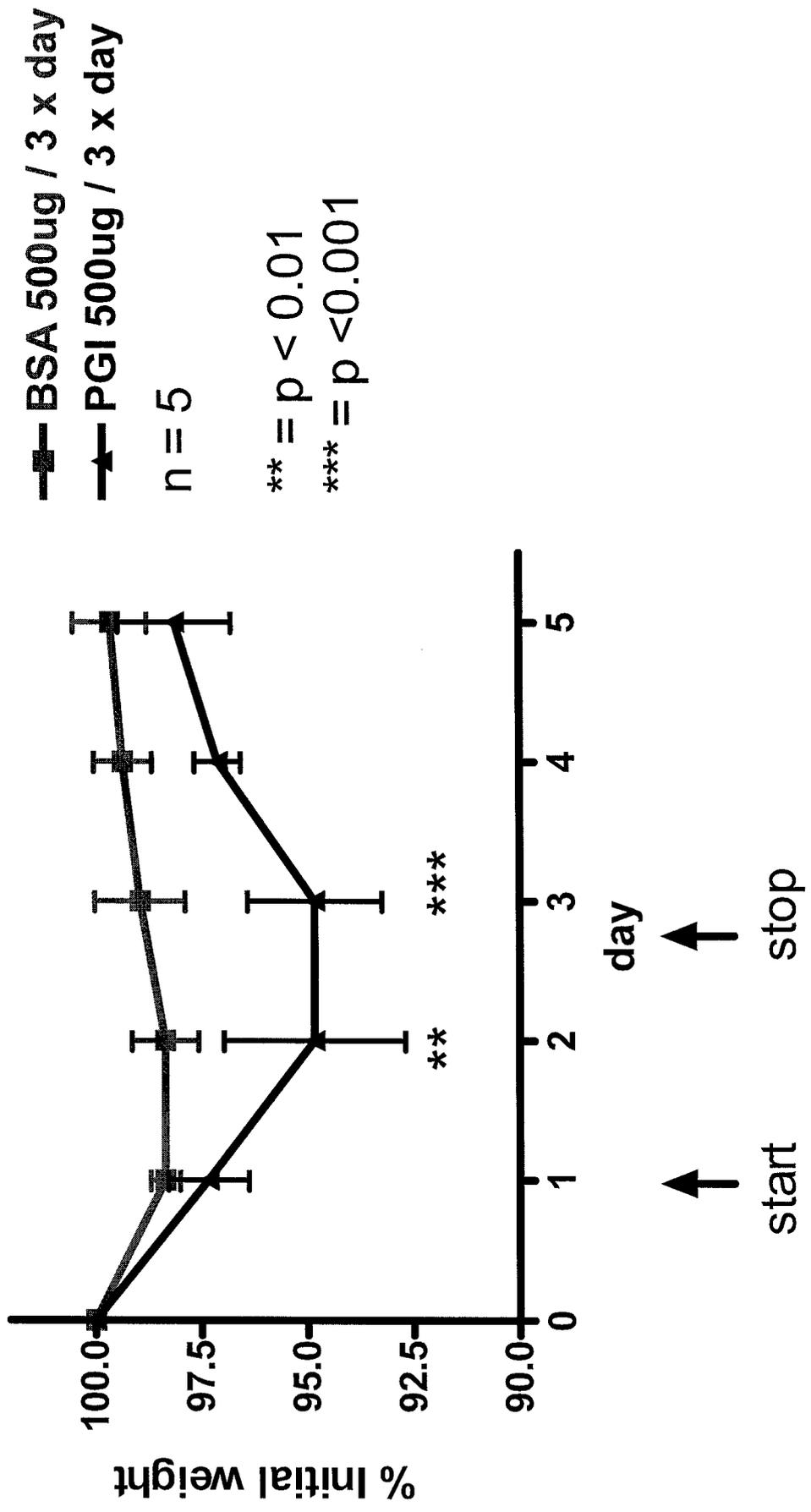
Summary - PGI & cachexia

- Cachectic mice have $\Uparrow$ plasma PGI

- No detectable serum IL-6 or TNFα

- PGI-secreting intramuscular CHO cells cause cachexia

- High-dose PGI injections $\downarrow$ wt 5%

- *Effect on muscle by adjacent tumor & high local [PGI]?*

- *Stimulation of muscle proteasomes?*

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Endothelin Axis Is a Target of the Lung Metastasis Suppressor Gene *RhoGDI2*

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Departments of [1]Molecular Physiology and Biological Physics, [2]Pathology, [3]Health Evaluation Sciences, Division of Biostatistics, and [4]Medicine, Division of Endocrinology, University of Virginia, Charlottesville, Virginia; and [5]Genomics Institute of the Novartis Research Foundation, San Diego, California

Abstract

Half of patients treated for locally advanced bladder cancer relapse with often fatal metastatic disease to the lung. We have recently shown that reduced expression of the GDP dissociation inhibitor, RhoGDI2, is associated with decreased survival of patients with advanced bladder cancer. However, the effectors by which RhoGDI2 affects metastasis are unknown. Here we use DNA microarrays to identify genes suppressed by RhoGDI2 reconstitution in lung metastatic bladder cancer cell lines. We identify such RNAs and focus only on those that also increase with tumor stage in human bladder cancer samples to discover only clinically relevant targets of RhoGDI2. Levels of endothelin-1 (ET-1), a potent vasoconstrictor, were affected by both RhoGDI2 reconstitution and tumor stage. To test the hypothesis that the endothelin axis is important in lung metastasis, lung metastatic bladder carcinoma cells were injected in mice treated with the endothelin receptor–specific antagonist, atrasentan, thereby blocking engagement of the up-regulated ET-1 ligand with its cognate receptor. Endothelin antagonism resulted in a dramatic reduction of lung metastases, similar to the effect of reexpressing RhoGDI2 in these metastatic cells. Taken together, these experiments show a novel approach of identifying therapeutic targets downstream of metastasis suppressor genes. The data also suggest that blockade of the ET-1 axis may prevent lung metastasis, a new therapeutic concept that warrants clinical evaluation. (Cancer Res 2005; 65(16): 7320-7)

Introduction

An estimated 53,000 new cases of bladder cancer are diagnosed in the United States each year. Approximately 5% of these patients present with metastatic disease at diagnosis. However, an additional 50% of patients who are originally diagnosed with locally advanced disease later develop metastases, commonly to the lung, resulting in significant mortality among patients with bladder cancer (1, 2). The molecular basis of pulmonary metastases is not well understood but is likely to involve the progressive loss of suppressor genes and dominant gain of function mutations in critical pathways along steps of the metastatic cascade (3). Whereas in theory, the metastatic cascade could be interrupted at various points with equal potential for therapeutic benefit, only

the steps that prevent the outgrowth of micrometastasis into a clinically life-threatening lesion are viable therapeutic targets. This is because the patients at greatest risk of metastatic disease are those diagnosed with clinically localized disease in which disseminated cancer cells may already be present in distant organs.

We recently showed that the GDP dissociation inhibitor, RhoGDI2, is a metastasis suppressor in a bladder cancer lung metastasis model (4). Metastatic derivatives of the bladder carcinoma cell line, T24 (termed T24T), were shown to exhibit loss of RhoGDI2, which correlated with increasing pathologic cancer stage and grade. Reexpression of RhoGDI2 in these deficient cells led to a dramatic suppression of lung metastasis. The clinical importance of RhoGDI2 loss of function was validated by showing that reduced or absent RhoGDI2 expression is strongly correlated with the development of metastasis, resulting in decreased survival following therapy for locally advanced bladder cancer (5). Because targeted clinical restoration of RhoGDI2 function is currently impractical, we used our isogenic metastatic cell model to identify genes regulated downstream of RhoGDI2 activity, whose encoded proteins may be pharmaceutically tractable. Using DNA microarrays to monitor the changes in gene expression following restoration of RhoGDI2 expression, we identified several potentially targetable proteins, including the endothelin-1 ligand (ET-1), that were suppressed in the presence of RhoGDI2 protein. These results suggest that loss of RhoGDI2 during the clinical progression of bladder carcinoma may lead to the up-regulation of the endothelin axis. This finding was confirmed by examining the relationship between RhoGDI2 expression levels and those of ET-1 in human tumor samples and cell lines. Collectively, our findings indicate that adjuvant trials with endothelin antagonists may be considered for patients with advanced bladder cancer following therapy of the primary lesion. In addition, application of this approach to other metastasis suppressor genes could identify potential novel targets for therapy in other common malignancies (6, 7).

Materials and Methods

Gene expression profiling of bladder tumor cell lines and human bladder cancers. Isogenic T24T cells, bladder carcinoma–derived cell lines, primary human bladder carcinomas, and normal bladder tissues were profiled on HG-U133A GeneChip arrays (Affymetrix, Santa Clara, CA). For the previously described isogenic T24T cell pair containing vector or RhoGDI2 constructs (4), duplicate RNA samples were generated from independent cell cultures; RNA from the 41 human bladder cancer cell lines [acquired from American Type Culture Collection (Manassas, VA) or from Dr. Monica Liebert (M.D. Anderson Cancer Center, Houston, TX)] was generated from a single log-phase culture. Following approval of the University of Virginia Human Investigation Committee, 23 primary bladder cancer samples of varying pathologic stages and five samples of normal urothelium were obtained and evaluated by a genitourinary pathologist (H.F.F.). For the malignant samples, only areas containing

>80% cancer cells were processed for RNA isolation. RNA was processed as previously described and hybridized to HG-U133A GeneChip arrays (Affymetrix). Image files were assessed for quality and artifacts and processed using Microarray Analysis Suite 5.0 (MAS 5.0, Affymetrix) using a scaling factor of 200.

Genes associated with RhoGDI2 expression in T24T cells were identified using the local-pooled error (LPE) test (8) with a false discovery rate (FDR) $P < 0.05$. Functional relationships among genes correlated with RhoGDI2 expression were analyzed using the ontology use in dCHIP1.3/ChipInfo (9, 10). The P value generated by this method indicates the strength of the association of gene clusters to the gene ontology terms or pathways, with $P < 0.05$ considered significant. We sought to identify genes encoding secreted proteins using two parallel approaches as previously described (11). To identify which of the 20 RhoGDI2-suppressed genes exhibited expression correlated with tumor stage, tumors with stage Ta were compared with tumors of stage T1 and above; these were then ranked according to fold change.

Neuromedin-U and endothelin axis evaluation in bladder cancer cells. T24T cells stably transfected and expressing RhoGDI2 transgene (T24T-RhoGDI2) or vector control (T24T-pcDNA3) were washed with PBS and total genomic RNA isolated and used as template for reverse transcription-PCR (RT-PCR) using primers for Neuromedin-U (NmU), ET-1, ET-1-converting enzyme, endothelin A receptor, and endothelin B receptor selected using OligoLite primer analysis software. Amplification was normalized to glyceraldehyde-3-phosphate dehydrogenase. A Qiagen One-step RT-PCR kit was used following the manufacturer's instructions. T24T, T24T-RhoGDI2, or T24T-pcDNA3 were plated and grown to 80% confluence in DMEM/F-12 + 5% fetal bovine serum. Then, the plates were washed with PBS and 14 mL DMEM/F-12 without serum was added. Cells were incubated in the above conditions for 36 hours. Medium was removed from the cells and analyzed by commercial ELISA assay (R&D Systems, Inc., Minneapolis, MN) according to the supplier's instructions.

Phospho-Erk1/2 evaluation in RhoGDI2-transfected cells stimulated with Endothelin-1. HOst, T24T-RhoGDI2, or T24T-pcDNA3 were serum starved overnight. Before lysis, cells were subjected to a 20-minute exposure of vehicle or varying concentrations of ET-1 (Sigma, St. Louis, MO). HOst cells were obtained from Cambrex (Walkersville, MD) and are Normal Human Osteoblasts (http://www.cambrex.com). Cells were then washed on ice in BioPlex Cell Wash Buffer followed by addition of 300 µL of BioPlex Lysis Buffer (containing phenylmethylsulfonyl fluoride and BioPlex phosphatase inhibitors), scraped and agitated on a microplate shaker. Protein concentration was determined by bicinchoninic acid assay buffer (Pierce, Rockford, IL) and allowed to incubate with phospho-Erk1/2-coupled beads (Bio-Rad, Richmond, CA) overnight. The lysate/bead mixture was then washed and exposed to phospho-Erk1/2 detection antibody and then allowed to incubate with Streptavidin-PE for 10 minutes, in the dark. Captured lysates were washed again and resuspended in BioPlex Resuspension Buffer before analysis. All assay samples were run in multiple biological replicates and tested in duplicate.

In vitro **and** *in vivo* **growth and metastasis assays.** Growth and colony formation in soft agar of T24T-RhoGDI2 or T24T-pcDNA3cells was analyzed ± atrasentan using techniques previously described (4, 12). S.c. tumorigenicity in 6-week-old nude mice was evaluated as described (13) with 5×10^6 cells in 0.1 mL of SFM. For experimental metastasis, mice were given an i.v. lateral tail vein injection with 10^6 tumor cells suspended in 0.1 mL of SFM as described (13). At the time of euthanasia, the lungs were removed by dissection away from adjacent organs and examined grossly and microscopically as described (4). RhoGDI2 immunohistochemistry on lung samples was carried out and scored as described (5) without knowledge of the experimental groups (atrasentan or vehicle/pcDNA3 or RhoGDI2) by the pathologist (H.F.F). The presence, number, and size of metastatic deposits were evaluated. For both s.c. tumorigenicity and experimental metastasis experiments, animals were separated into two groups: group 1, pcDNA3 transfected cells and group 2, pcDNA3 + atrasentan. Mice were treated with either atrasentan (5 mg/kg/d) in their drinking water or not (control water) for 12 weeks. This dose of atrasentan has previously been shown to give effective serum concentrations of the drug in nude mice (14). *In vivo*

experiments were carried out twice or more and results pooled for the statistical analysis.

Statistical methods. Expression levels were transformed to the natural log scale to stabilize the variance. The Pearson correlation coefficient was used to assess the relationship between the log ratios of ET-1 to RhoGDI2 and NmU to RhoGDI2. F tests were used to assess trends in the fold changes in HOst, pcDNA3, and RhoGDI2 with the addition of varying levels of ET-1. χ^2 tests were used to compare the proportion of mice developing metastases in the pcDNA3, RhoGDI2 and the pcDNA3 + atrasentan groups. For each group, exact 95% confidence intervals based on the binomial distribution were used for the proportion of mice with metastases.

Results

Previous microarray analysis of a nonmetastatic human bladder cancer cell line, T24 and a lineage-related lung metastatic variant, T24T, revealed the loss of expression of several genes, including the GDP dissociation inhibitor, *RhoGDI2*. Following reexpression in T24T cells, RhoGDI2 functions as a potent suppressor of lung metastasis (4). We have also shown that *RhoGDI2* gene expression correlates with metastatic competence in bladder carcinoma–derived cell xenografts and have shown here that RhoGDI2 protein expression is also decreased as a function of increased metastatic capability in a series of human bladder cell lines (Fig. 1A). To begin to understand the mechanism by which RhoGDI2 suppresses metastasis, we reasoned that genes downstream of RhoGDI2 activity in bladder cancer patients may be down-regulated by reexpression of RhoGDI2 in RhoGDI2-deficient, metastatic cells. To determine the identity of these genes, stable T24T derivative bladder cancer cells were selected with empty vector or a construct expressing RhoGDI2 (Fig. 1A). RNA from these cells was hybridized to a microarray (11) containing ~21,500 human genes. Sixty-three and 40 genes were up-regulated and down-regulated by >2-fold, respectively, in RhoGDI2-expressing cells. Analysis of the data with respect to gene sequence and GO annotations indicated that several of these encode putatively secreted proteins that may be involved in autocrine or paracrine signaling (Table 1). An ontological view of these genes (Table 2) revealed that many are involved in cytoskeletal organization providing potential insight into the mechanism of action of RhoGDI2.

To determine which of the 40 down-regulated genes in stable RhoGDI2 T24T transfectants may be clinically relevant targets, we searched for genes whose expression was increased as a function of tumor stage in a series of 20 human bladder cancers using microarray analysis. Tumor tissue from lung metastases is not available from bladder cancer patients. This is primarily because removal of metastases in patients with bladder cancer is not required for routine medical care. Therefore, we evaluated gene expression from primary human bladder carcinomas according to pathologic stage, as the frequency of metastasis robustly increases with higher tumor stage (15–17). Whereas 28 genes were up-regulated in association with increasing stage, only two of these were also down-regulated by stable RhoGDI2 expression in the cell line model (Table 1). The relationship between expression of these two genes and tumor stage is shown in Fig. 1B. In the T24T cell line system, the most strongly down-regulated mRNA in response to RhoGDI2 was ET-1, which was decreased 9.5-fold (Table 1), whereas NmU was decreased 3-fold. These microarray expression levels were independently verified by RT-PCR analysis (Fig. 1C). As ET-1 is a secreted factor that requires enzymatic processing to generate the mature active form by endothelin-converting enzyme (ECE), we tested conditioned media from both RhoGDI2 stably transfected and vector control cell lines for the presence of active ET-1 protein.
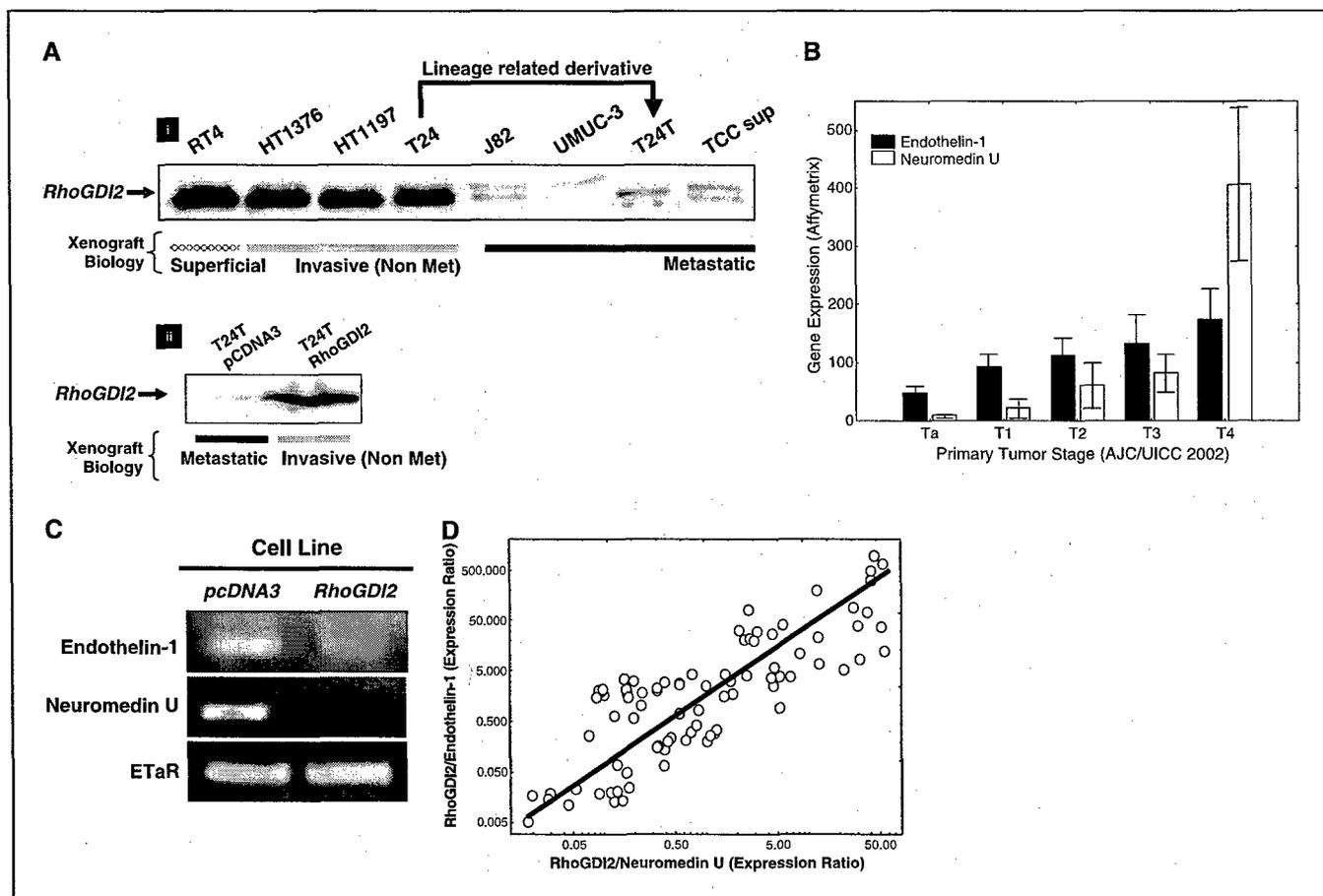
Figure 1. *A,* expression of RhoGDI2 protein in human bladder cell lines as a function of clinical and xenograft behavior. *i,* cell lysates of bladder cancer cell lines were prepared and Westerns probed with anti-RhoGDI2 antibody carried out as described (4). Xenograft behavior is indicated using color-coded lines. *ii,* protein expression of T24T cells stably transfected and expressing RhoGDI2 transgene or vector control (pcDNA3). *B,* ET-1 and NmU mRNA expression as a function of human bladder cancer stage. A set of 23 primary carcinomas (five Ta, five T1, six T2, three T3, and four T4) was macrodissected and areas comprised of >80% viable carcinoma was used for expression profiling using the Affymetrix HG-U133A array as described. *Columns,* mean Affymetrix expression levels as a function of stage; *bars,* SD. Both Et-1 ($P = 0.004$) and NmU ($P = 0.001$), which show significant increases in expression levels across stage, assuming a linear trend in the ln(expression level). *C,* expression of ET-1, NmU, and endothelin receptor A (ETa) in RhoGDI2- and vector-transfected cells. RT-PCR was done with primers specific for NmU and ET-1 as described in Materials and Methods. The template used was RNA isolated from T24T-RhoGDI2 and T24T-pcDNA3 cells. *D,* expression ET-1 and NmU as a function of RhoGDI2 in bladder cell lines. Forty-one human bladder cell lines were analyzed on oligonucleotide arrays for the expression levels of RhoGDI2, NmU, and ET-1. Ratio of NmU to RhoGDI2 expression (*X-axis*); ratio of endothelin to RhoGDI2 expression (*Y-axis*). Regression line is shown. Correlation = 0.85; 95% confidence interval, 0.78-0.90; $P < 0.001$.

In RhoGDI2-expressing cells, ET-1 was present at a concentration of 7 pg/mL, whereas in vector control cells, ET-1 was present at 69 pg/mL, showing that RhoGDI2 affects the active levels of this secreted peptide.

To further explore the relationship between RhoGDI2 expression with that of ET-1 and NmU in bladder cancer, microarray data from 41 human bladder cancer cell lines were viewed as a logarithmic plot of the ratio of ET-1 to RhoGDI2 versus ratio of NmU to RhoGDI2. The log-linear relationship suggests a codependent relationship between RhoGDI2 and both ET-1 and NmU expression (Fig. 1D; correlation between log ratios, 0.85; 95% confidence interval, 0.78-0.90; $P < 0.001$). Taken together with data from the RhoGDI2 transfection studies in T24T cells and the human bladder carcinoma data, this finding independently supports and generalizes the importance of RhoGDI2 regulation of ET-1 and NmU in human bladder cancer.

The endothelin axis is composed of several members that can be expressed by both cancer cells and host cells resulting in both autocrine and paracrine interactions favoring growth and angio-

genesis (18). Hence, we sought to evaluate this axis by examining the microarray data and confirming these findings by RT-PCR analysis. These data showed that vector-transfected T24T cells express mRNAs for the two endothelin receptors, endothelin A receptor (ETaR) and endothelin B receptor (ETbR), as well as the ECE. Although mRNA expression of these three genes was uniformly lower in RhoGDI2-transfected cells, this did not reach statistical significance (data not shown). Because we had previously established that ET-1 is produced in the medium conditioned by the vector-transfected cells, we sought to determine whether this ET-1 plays a role in cell growth *in vitro* and *in vivo*, using the selective ETaR antagonist, atrasentan. ETaR blockade was shown to have no effect on growth in monolayer culture (Fig. 2A) or to affect the anchorage-independent colony forming ability of RhoGDI2-stable or empty vector transfected cells (Fig. 2B). In addition, no difference in local tumor growth was observed following s.c. injection of immunocompromised mice with vector and RhoGDI2-transfected T24T cells and treated with atrasentan or vehicle for 8 weeks.

ET-1 is known to induce cell proliferation and differentiation through multiple G-protein-linked signaling systems (14). Studies have also shown that ET-1 induces a biphasic activation of p21ras, which subsequently activates the extracellular signal-regulated kinase (Erk) cascade (19). Our cumulative data above shows that blockade of the autocrine endothelin axis does not have an effect on *in vitro* growth or colony formation in bladder cancer cells, suggesting the possibility that these cells are insensitive to ET-1 by virtue of receptor loss or defects in signaling. However, as shown above (Fig. 1*C*), these cells do express the ET-1 receptor, ETaR. Evaluation of the effect of ET-1 on Erk phosphorylation revealed sensitivity of these cells to ET-1. In contrast, reexpression of RhoGDI2 resulted in a marked blockade of this effect, suggesting that this protein blocks the

Table 1. Gene expression differences found with Affymetrix HG-U133A GeneChip array analysis of pcDNA- and RhoGDI2-transfected metastatic human bladder cancer cell lines

Probe set	Gene*	Fold difference[†]
Expression lost with RhoGI2 reexpression		
218995_s_at	*ET-1*[‡]	−9.53
221729_at	*Collagen, type V, α2*[‡]	−7.49
201669_s_at	Myristoylated alanine-rich protein kinase C substrate (*MARCKS*)	−6.53
217929_s_at	Hypothetical protein *FLJ14225*	−5.08
208893_s_at	*Dual specificity phosphatase 6*	−3.93
204036_at	*Lysophosphatidic acid G-protein-coupled receptor*	−3.70
220779_at	*Peptidylarginine deiminase type III*	−3.64
201012_at	Annexin A1 (*ANXA1*)	−3.61
208906_at	Similar to *G-protein γ3 linked gene*	−3.59
205125_at	Phospholipase C, delta 1 (*PLCD1*)	−3.48
213005_s_at	*KIAA0172* gene	−3.47
206023_at	*NmU*[‡]	−3.01
221797_at	Clone *CDABP0028* mRNA sequence	−2.94
200897_s_at	Palladin (*KIAA0992*)	−2.84
202388_at	Regulator of G-protein signaling 2 (*RGS2*)	−2.80
201015_s_at	Plakoglobin (*JUP*)	−2.65
201952_at	*Activated leucocyte cell adhesion molecule*	−2.48
215785_s_at	*p53-inducible protein*	−2.39
214463_x_at	H4 histone family, member D (*H4FD*)	−2.37
209791_at	Orthologue of mouse and rat PDI (*peptidylarginine deiminase*)	−2.35
Expression gained with RhoGI2 reexpression		
219424_at	EBV0induced gene 3 (*EBI3*)[‡]	6.52
206026_s_at	Tumor necrosis factor, α-induced protein 6 (*TNFAIP6*)[‡]	4.39
217077_s_at	*G-protein-coupled receptor 51*	4.22
205844_at	Vanin 1 (*VNN1*)	4.07
205973_at	Fasciculation and elongation protein zeta 1 (*zygin I*)	3.69
216384_x_at	Prothymosin α (*PTMA*)	3.65
209991_x_at	Orphan G-protein-coupled receptor HG20 (*HG20*)	3.62
221660_at	*Myosin light chain 2, precursor lymphocyte-specific, clone*	3.45
209008_x_at	*Cytokeratin 8*	3.23
211003_x_at	*Transglutaminase 2*	3.15
219486_at	Hypothetical protein *FLJ20399*	2.92
218644_at	Pleckstrin 2 (mouse) homologue (*PLEK2*)	2.91
201042_at	*Transglutaminase 2*	2.91
205433_at	Butyrylcholinesterase (*BCHE*)	2.90
210155_at	*Myocilin*[‡]	2.85
204642_at	Sphingolipid G-protein-coupled receptor 1 (*EDG1*)	2.80
221477_s_at	*Clone MGC:5618*	2.70
203180_at	Aldehyde dehydrogenase 1 family, member A3 (*ALDH1A3*)	2.68
202350_s_at	Matrilin 2 (*MATN2*) precursor	2.64
211573_x_at	*Transglutaminase mRNA*	2.64

NOTE: Sixty-three genes were up-regulated with RhoGDI2 reconstitution in T24T cells, whereas 40 genes were down-regulated. Top 20 of each group are shown.
*All genes shown have $P < 0.0001$ for the LPE test for testing the null hypothesis of equal mean gene expression of pcDNA- and RhoGDI2-transfected cells.
[†]Fold change comparing pcDNA- with RhoGDI2-transfected cells.
[‡]Identified as a secreted factor based on algorithms described in Materials and Methods.

intracellular signaling pathways downstream of the ETaR (Fig. 2C). This finding is particularly relevant because it suggests that RhoGDI2 may block paracrine tumor-host crosstalk in distant organs such as the lung, where metastasis occurs. We tested this hypothesis below.

The endothelin axis, specifically ET-1, is known to be important for the regulation of multiple biological processes including angiogenesis in the normal lung (14, 20). Furthermore, ET-1 is a known angiogenic factor responsible for the tumor vasculature of cancers that metastasize to the lung (14, 21). Taken together with the results presented above, we reasoned that the paracrine effects of pulmonary ET-1-coupled with the autocrine effects of tumor ET-1 stimulate the growth of T24T cells in the lung. Conversely, by their decreased expression and lack of responsiveness to ET-1, T24T cells with reconstituted RhoGDI2 can no longer form metastases.

To test this hypothesis, we injected stably transfected T24T vector or RhoGDI2 overexpressing cells into the tail veins of ten 8-week-old nude mice, which were subsequently exposed to drinking water containing atrasentan or vehicle. After 8 weeks, the lungs were inspected grossly and microscopically (Fig. 3A). Fifty-three


Figure 2. A, role of the ETaR antagonist atrasentan on the *in vitro* growth monolayer of T24T cells. Growth curves in monolayer culture of T24T-RhoGDI2 and T24T-pcDNA3 cells treated with the selective ETaR antagonist atrasentan or vehicle for 5 days. *Point,* average measurements of five replicate wells; *bars,* SD. Representative of assays carried out three independent times. B, role of ETaR antagonist atrasentan on colony formation in agar of T24T cells. Soft agar cultures were analyzed after 3 weeks growth in agarose with either two different concentrations of atrasentan or control water. C, phospho-Erk1/2 evaluation in RhoGDI2-transfected cells in response to ET-1 stimulation. HOst, T24T-RhoGDI2, and T24T-pcDNA3 cells were serum starved overnight. Before lysis, cells were subjected to a 20-minute exposure of vehicle or varying concentrations of ET-1. Lysates and analysis was prepared according to Bio-Rad Bio-Plex Phosphoprotein testing instructions as described in Materials and Methods. Results are pooled data from two biological replicates with duplicate samples in each. Significant changes in expression compared to vehicle control are observed with HOst ($P < 0.001$) and pcDNA ($P < 0.001$) but not with RhoGDI2 ($P = 0.73$). *Columns,* means; *bars,* SD.

Table 2. Gene Ontology Analysis based on Affymetrix HG-U133A GeneChip array analysis of pcDNA- and RhoGDI2-transfected metastatic human bladder cancer cell lines

Ontology class	RhoGDI2*	All genes	$P^{\dagger}$
Calmodulin binding	5	$28^{\ddagger}$/11,336§	0.000
Cytoskeletal protein binding	5	149/11,336	0.000
Cell motility	6	475/11,336	0.000
Actin cytoskeleton	4	202/11,336	0.001
GTPase activator	3	93/11,336	0.001
Extracellular matrix glycoprotein	2	32/11,336	0.002
Signal transducer	11	2209/11,336	0.003
G-protein-coupled receptor protein signaling pathway	5	490/11,336	0.003
Signal transduction	11	2334/11,336	0.005
Small GTPase regulatory/ interacting protein	3	191/11,336	0.007
Enzyme activator	3	192/11,336	0.007
Cell surface receptor linked signal transduction	6	868/11,336	0.008
Receptor signaling protein	4	419/11,336	0.011
Enzyme regulator	4	442/11,336	0.013
Ligand	4	453/11,336	0.014
Plasma membrane	10	2397/11,336	0.018
Protein binding	6	1048/11,336	0.019
Cytoskeleton	4	504/11,336	0.020
Cell communication	13	3688/11,336	0.023
Soluble fraction	3	303/11,336	0.025

*Number of genes found to be significantly changed (increased or decreased) as a function of RhoGDI2 in the corresponding ontology class.

†Calculated with dCHIP v1.3. Values < 0.05 considered significant for association of genes to the gene ontology terms or pathways discovered.

‡Number of genes with specific annotated ontology on entire chip.

§Number of genes with annotated ontology on entire chip.

percent of the mice receiving the stable T24T-empty vector cells developed lung metastases. This was reduced to 5% by treatment with atrasentan. Mice exposed to RhoGDI2-overexpressing cells had a 20% incidence of lung metastases. In control mice, the tumor size of metastases ranged from 0.8 to 6 mm compared with 1.3 to 1.5 mm for metastases of RhoGDI2-transfected cells and 0.8 to 1.0 mm size of metastases in atrasentan-treated mice. Among the mice who developed at least one metastasis, the mean number
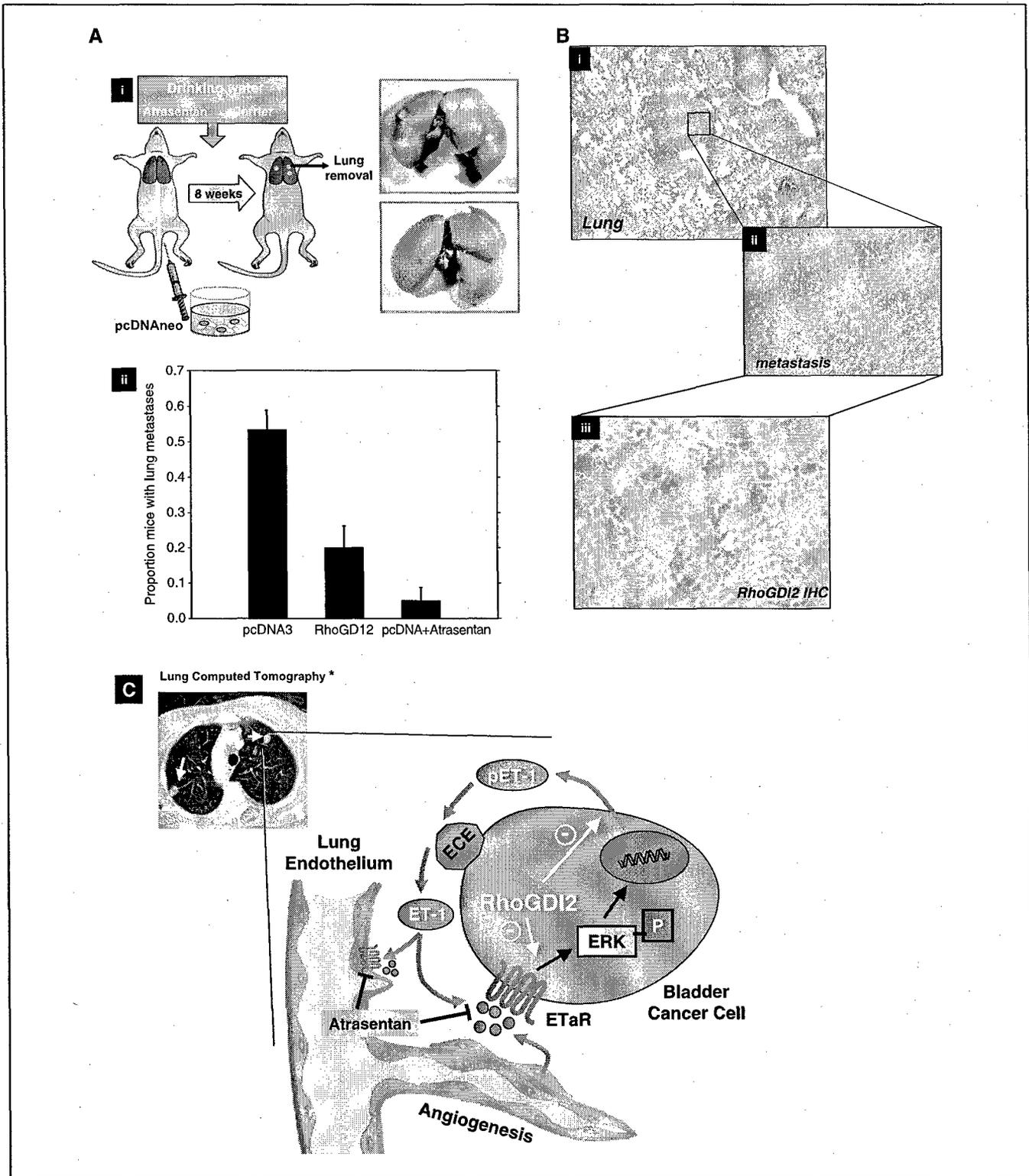
Figure 3. *A, in vivo* lung metastasis in mice injected with bladder cancer cells. *i,* Mice in each of two groups were injected s.c. with T24T-RhoGDI2 and T24T-pcDNA3 cells. After randomization, one group of mice received atrasentan in their drinking water, the other group received vehicle. Animals were euthanized at 12 weeks and lungs examined grossly and then processed for microscopy and immunohistochemistry as described in Materials and Methods. *ii,* a plot of incidence of lung metastasis from four independent experiments. *Columns,* means; *bars,* SD. The groups are significantly different with respect to the proportion of mice with lung metastases ($P < 0.0001$, χ^2 test). *B,* histologic appearance of lung metastases: H&E-stained sections [*H&E*, ×40 (*i*) and ×200 (*ii*)] and immunohistochemical (*iii*) staining for RhoGDI2 in pulmonary metastases obtained from RhoGDI2-transfected T24T bladder cancer cells injected i.v. via tail vein. Avidin-biotin immunoperoxidase technique described in Materials and Methods is shown at ×400 magnification. *C,* diagrammatic representation of the autocrine and paracrine effects of RhoGDI2 on the endothelin axis in bladder cancer lung metastasis. Abbreviation: preproendothelin-1, pET-1.

of metastases per mouse was 25.3 (range, 20-80) in the control group, 3.4 (range, 0-6) for those with RhoGDI2-overexpressing cells, and 1.0 (range, 0-2) in the group treated with atrasentan. Overall, this indicates that the effect of atrasentan in suppressing metastasis mediated by the loss of RhoDGI2 is similar to reexpression of the gene itself. Careful microscopic examination of cancer cell morphology, percent tumor necrosis and location of metastatic foci relative to the vasculature did not differ between groups (Fig. 3*B, i* and *ii*; data not shown). Immunohistochemical analyses of lung metastases in mice with RhoGDI2-transfected T24T cells using an anti-RhoGDI2 antibody (Fig. 3*B, iii*) showed that ~70% to 90% of the tumor cells expressed the protein. This observation suggests that the cells may have developed compensatory mechanisms to overcome the metastatic suppression by RhoGDI2 by loss of downstream mediators of RhoGDI2 function or other pathways.

Discussion

We previously identified RhoGDI2 as a putative suppressor of metastasis in an isogenic model of bladder cancer metastasis. The clinical importance of this observation was underscored by the fact that reduced RhoGDI2 expression was found to be associated with decreased survival for patients with bladder cancer. In the present study, we postulated that RhoGDI2 works as a brake on the expression of prometastatic genes and sought to identify such genes in a combined analysis of the T24/T24t cell model and primary human bladder carcinomas. Because clinical reconstitution of RhoGDI2 protein is currently impractical, we sought to discover clinically druggable proteins or pathways downstream of RhoGDI2. We required these genes to be up-regulated following the loss of RhoGDI2 protein from the cells and be capable of being inhibited by small molecule antagonists or functionally antagonistic antibodies.

Precedence for the inhibition of cellular activities downstream of tumor suppressor gene loss is limited but informative. A key example is the constitutive activation of AKT and mammalian target of rapamycin (mTOR) in PTEN-deficient cells. Inhibition of mTOR kinase by rapamycin has been shown to lead to a loss of cell viability, and there is evidence that inhibitors of AKT phosphorylation may have the same potential (22). Although not particularly tractable from a therapeutic stand point, up-regulation of E2F proteins following loss of pRB and stabilization (23) of β-catenin and therefore β-catenin/TCF-4-mediated transcription are also important examples of up-regulated genes and pathways that become activated following the loss of tumor suppressor genes. Here, we show that the inhibition of the endothelin axis, mediated by the blockade of up-regulated functional ET-1 ligand, leads to metastatic suppression in cells deficient for RhoGDI2. Indeed, inhibition of the endothelin axis is particularly attractive, because we find no evidence for loss of organismal or tissue viability.

The endothelin ligand has been shown to regulate vascular tone, tissue differentiation, cell proliferation, hormone production, cell invasion, angiogenesis, and bone remodeling (18). ET-1 also has potent effects on cells in the skeleton (18) and lung (24), two important sites of bladder cancer metastasis (15). In addition to such direct effects, ET-1 may indirectly increase vascular endothelial growth factor (VEGF) and induce hypoxia-inducible factor 1 α (25). ET-1 and VEGF stimulate one another (26), resulting in

proliferation of endothelial and vascular smooth muscle cells thus promoting tumor growth. Previous work also shows a role for tumor-secreted ET-1 in skeletal metastases from breast and prostate cancers (14) and supports a cyclical model in which tumor-secreted ET-1 stimulates bone cells, in turn providing a fertile microenvironment for metastases (27). A similar cycle could occur in other in endothelin-responsive tissues, such as the lung and kidney (24, 28) and may be the primary driving force for lung metastasis. Further evidence for this model comes from the observation that neither RhoGDI2 reconstitution nor atrasentan treatment altered primary tumor growth, suggesting that both act at the metastatic site, likely in breaking paracrine signaling (Fig. 3*C*).

The molecular relationship between ET-1 and RhoGDI2 shown here is highly significant, because of the existence of clinically tested, orally active small molecule ET_A antagonists such as atrasentan with good clinical safety profiles (14). In fact, 39 patients, 30 of whom had prostate cancer, were treated in a recently reported dose escalation trial. The most common adverse events were rhinitis, headache, and peripheral edema indicate that atrasentan is well tolerated, with no dose-limiting adverse events observed up to 95 mg (29). Furthermore, in other studies, quality of life was not adversely affected by atrasentan (30).

As yet, we do not know how RhoGDI2 suppresses ET-1 expression. RhoGDI2 may have similar targets to those modulated by RhoGDI1, such as RhoA, which affects the cytoskeleton and influences metastatic disease progression. RhoGDI2 may also prevent activation of RhoA and RhoB (31), which stimulate the ET-1 promoter (32). Interestingly, levels of RhoA and RhoC have been reported as predictors of metastasis for patients with bladder cancer (33, 34). Our data suggests that ET-1 blockade may interfere with tumor-host interactions in the lung, thus endothelin receptor antagonists, such as atrasentan. Such drugs could reduce the development of lung metastases following resection of advanced bladder cancers by blocking the growth of a micrometastasis, which during its early developmental phase, is highly dependent on the host organ ET-1 for growth. Furthermore, because ET-1 stimulates smooth muscle cells (35), ET_A antagonists might also inhibit muscle invasive primary bladder cancer, which could reduce patient morbidity from radical cystectomy. Such ET_A antagonism could be tested in a model of muscle-invasive disease, which we have described (36, 37).

In conclusion, we have applied a novel approach to identify druggable targets which become activated following the loss of RhoGDI2 expression. We provide strong support for this concept *in vivo* by showing that an ET_A antagonist effectively decreased lung metastases in a bladder cancer animal model thus validating ET-1 as a novel therapeutic target in lung metastasis. These findings suggest that clinical inhibition of ET-1 activity might be considered in clinical trials of patients with bladder cancer seeking to reduce the frequency of relapse with pulmonary metastasis.

Acknowledgments

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